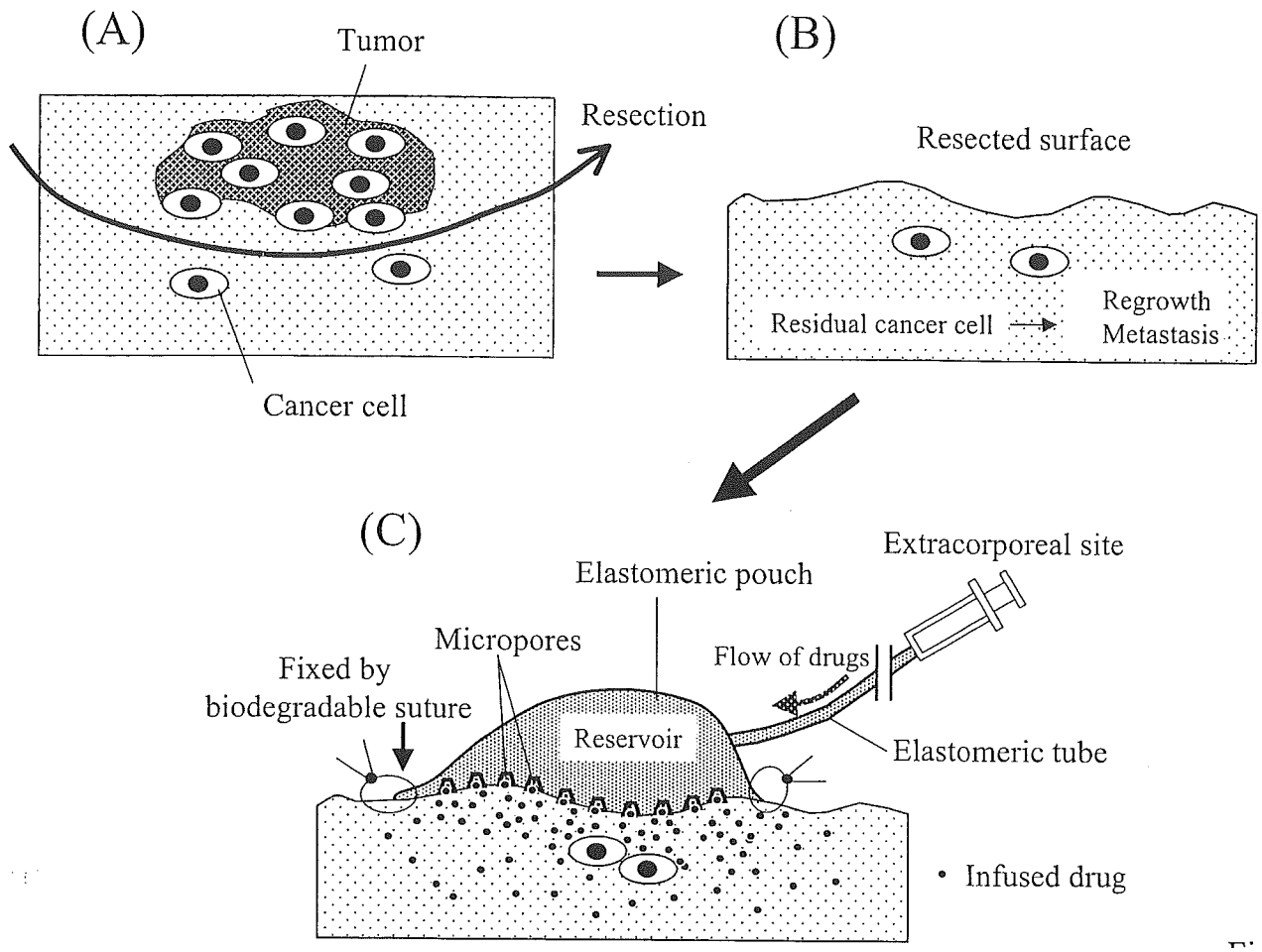

Figure 1. (A) Resection of pancreatic cancer tumor. (B) Regrowth and metastasis due to residual cancer cell in the remaining tissue. (C) Scheme of device-directed local infusion system. The pouch of the device could be fixed to the target tissue by biodegradable sutures. The elastomeric tube connected to the pouch is guided to an extracorporeal site, which enables the reloading of drugs and easy extraction of the device upon pulling the tube out.

2(A)]. The elastomeric pouch, made of segmented polyurethane (SPU), has high flexibility, which enables the drug-delivery surface of the device to tightly adhere to the resected surface by biodegradable sutures. The elastomeric tube (percutaneous infusion tube) enables the reloading of an additional dose from the extracorporeal site. The device can be easily pulled out with the tube because the sutures are biodegraded by the removal time and the pouch itself is very flexible so as to collapse mechanically, facilitating the removal of the device without appreciable difficulty.

In this article, to verify the therapeutic effectiveness of such a device in a clinical setting, an experiment was done using a mouse and an experimental infusion device for mice was fabricated for sustained infusion of a drug using an osmotic pump, instead of a percutaneous infusion tube. Gemcitabine (GEM, 2′,2′-difluorodeoxycitidine) was used as a therapeutic drug, because GEM is currently the most effective drug for pancreatic cancer.[14,15] Its high-suppressive effect on the growth of heterotopic human pancreatic tumor was noted in this study. On the basis of preliminary promising results and a prototype device, potential applicability in a clinical setting is discussed.

MATERIALS AND METHODS

Fabrication of the Prototype Model

The thin elastomeric film used for the elastomeric pouch is segmented polyurethane (SPU, Elatollan ET880; film thickness 100 μm; Sheedom Co., Ltd., Tokyo), which can be attached to resected tissues using biodegradable sutures. Multimicropores on the side of delivery [Figure 2(B)] were formed by a laser ablation technique using a KrF excimer laser (wavelength, 248 nm) microprocessing apparatus (L4500, C4540; Hamamatsu, Japan) operating with a computer-assisted design, according to our previous report (pore diameter 100 μm; pore-to-pore distance 500 μm). The microporous SPU film (approximately 1.5 × 1.0 cm) and nonmicroporous SPU film (1.5 × 1.0 cm) were glued using *N,N*-dimethylformamide (DMF) as a good solvent of SPU. This pouch was connected to the polyethylene tube (Alza Corporation, Palo Alto, CA). To the opposite end of the tube, an ALZET osmotic pump (Alza Co., 1007D, 0.5 μL/h for 7 days), as a drug infuser for an experimental small-animal model, was connected [Figure 2(C)].

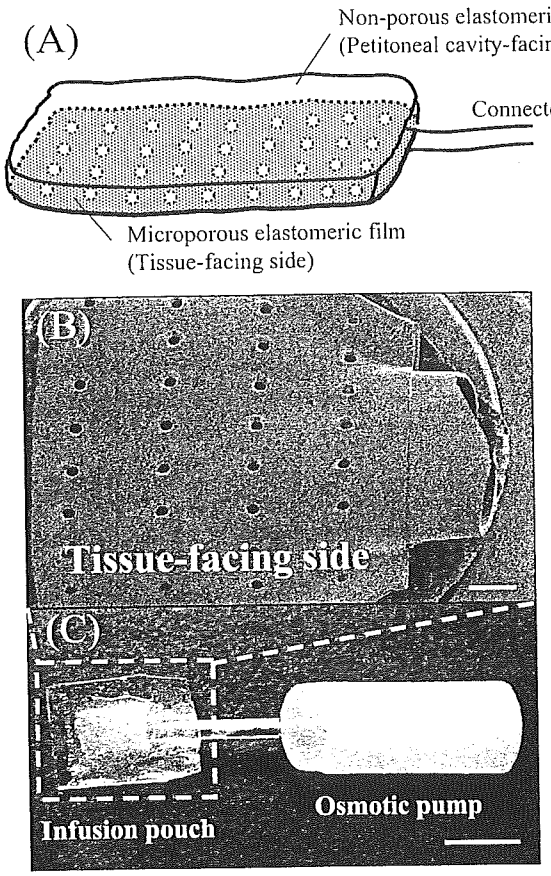

Figure 2. (A) Scheme of the infusion device. (B) Scanning electron microscopy of the tissue-facing surface of the pouch made of segmented polyurethane (SPU) shows micropores formed by a laser ablation technique. Bar: 1 mm. (C) The experimental device used for a nude mouse, which was connected to an ALZET osmotic pump by a polyethylene tube. Bar: 5 mm.

Subcutaneous Tumor Model and Therapy

AsPC-1,[16] a human pancreatic cancer cell line, was cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with streptomycin, penicillin and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. GEM was purchased from Eli Lilly and Co. (Indianapolis, IN). Five-week-old nude mice (BALB/c nu/nu, Kyudo Co., Ltd., Saga, Japan) were subcutaneously inoculated with 3×10^6 AsPC-1 cells/50 μL in the left flank. Three days after subcutaneous inoculation, the treated mice were treated in the following five groups (the detailed procedures are described in Table I): Group I (no treatment), Group II (weekly single intraperitoneal GEM injection), Group III (1-week local continuous infusion of GEM at a site away from the tumor), Group IV [1- week local continuous infusion of phosphate-buffered saline (PBS)], and Group V (1-week local continuous infusion of GEM). The devices in Group III were implanted in a site far remote from the tumor, but those in Groups IV and V were implanted just adjacent to tumors with biodegradable sutures on the tumor tissue. Each group consisted of six mice.

Two perpendicular diameters of resultant subcutaneous tumors were measured with calipers every 3 days, and the tumor volume was calculated using the formula: tumor volume (mm^3) = 0.52 × (width [mm])^2 × (length [mm]).

Statistical Analysis

Statistical analysis was performed using the analysis of variance (ANOVA). Post hoc comparisons were made by the Scheffe analysis. Differences were considered significant at $p < 0.05$.

RESULTS

The major part of infusion device is a rectangular-shaped pouch (1.5 × 1.0 cm) with a connector port, which was assembled by gluing two thin elastomeric films and a connector tube (Figure 2). The films were made of SPU. The tissue-facing film of the device is micropored by excimer laser-ablation technique (pore size; 100 μm in diameter, poor-to-pore distance; 500 μm) and the peritoneal cavity-facing film is nonporous. A human pancreatic cancer cell line (AsPC-1; 3×10^6) was subcutaneously inoculated for 3 days, followed by infusion device-directed therapy with GEM. In these particular animal models, infusion was driven by an osmotic pump, instead of extracorporeal drug infusion.

Upon injection of a large number of pancreatic cancer cells (AsPC-1) into subcutaneous tissue of mice, a tumor was established. These tumor-bearing mice were treated five different ways as described in detail in Table I. Figure 3 shows the time courses of the tumor volumes of these five groups after the initiation of therapy [Figure 3(A)] and the appearances of the

TABLE I. Different Therapeutic Procedures in Tumor-Bearing Tissues

Group	Treatment	Drug Administration	Duration
I	None	None	—
II	Weekly single intraperitoneal injection of GEM	50 mg/kg body weight/week (corresponding to the dosage of approximately 1 mg/body/week)	4 weeks (one per week)
III	Local continuous infusion of GEM (at the remote site from tumor tissue)	10 mg/kg body weight/week (200 μg/body/week)	1 week
IV	Local continuous infusion of PBS (at the site of tumor tissue)	None	1 week
V	Local continuous infusion of GEM (at the site of tumor tissue)	7.5 mg/kg body weight/week (150 μg/body/week)	1 week

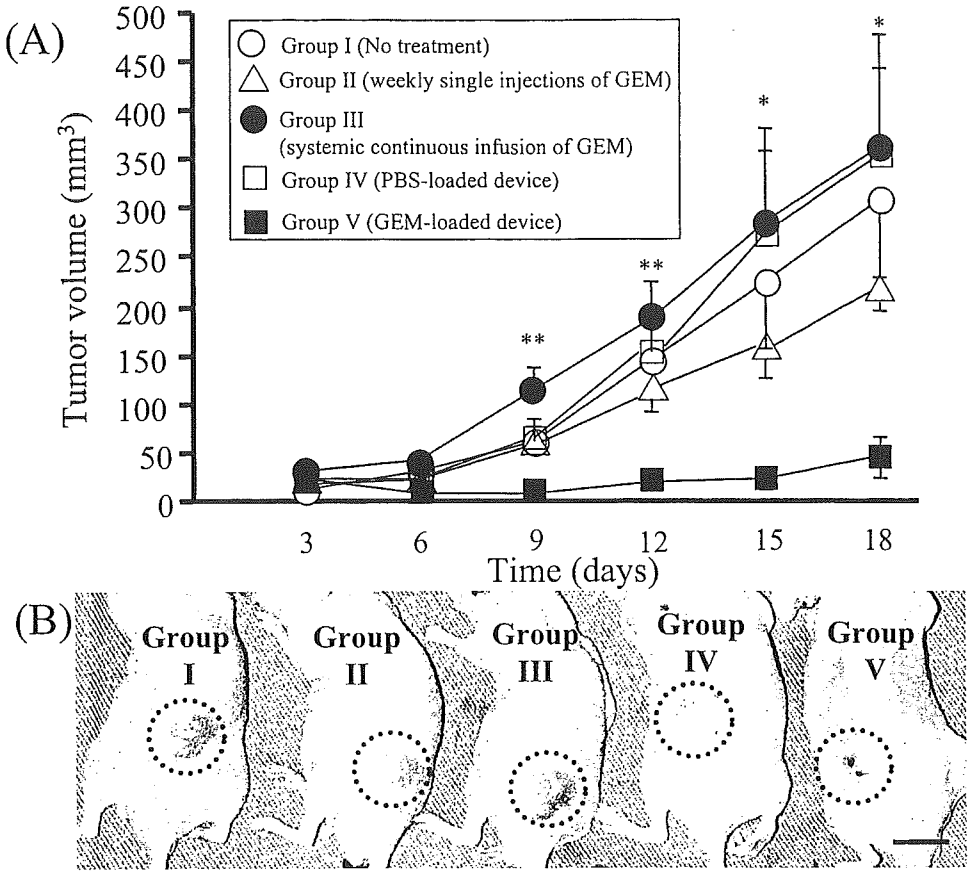

Figure 3. The growth of subcutaneously inoculated pancreatic cancer cell (AsPC-1). (A)Time course of the development of tumor arising from AsPC-1 cells. Values are expressed as means ± SE (n = 6). **Significant difference ($p < 0.05$) between Group V and all the other groups. *Significant difference ($p < 0.05$) between Group V and all other groups except for Group II. Because little significant change in the order of tumor volume between Groups was observed after 18 days of observation, the result after 18 days was deleted. (B) Typical gross appearance 12 days after AsPC-1 cell injection of each group. Bar: 10 mm.

heterotopic pancreatic tumors on day 12 [Figure 3(B)]. The tumors in Groups I (no treatment), II (weekly single intraperitoneal GEM injection), III (continuous GEM infusion at the remote site apart from a tumor tissue), and IV (continuous PBS-infusion device) rapidly grew with time. In contrast, in Group V (GEM infusion on the tumor) a significant suppression in pancreatic tumor volume was observed compared with all the other groups on days 9 and 12 and in all the other groups except for Group II on days 15 and 18 ($p < 0.05$). In four of the six mice in Group V, the tumors became macroscopically undetectable after day 6 and not noted at all over an observation period of 30 days. On the other hand, the tumors of the other two mice in Group V grew similar to those in the other groups after the therapy was completed on day 7. In all groups, a significant body weight loss was not observed.

DISCUSSION

The poor postoperative prognosis of pancreatic cancer is mainly due to a high incidence of relapse, such as local recurrence and hepatic metastasis.[5–8] Regional chemotherapy was reported to reduce the incidence of hepatic metastasis, which occurs from remnant pancreatic cancer cells via portal vein and makes hepatic tumors after surgery, and prolong the survival period;[17–20] however, no regional chemotherapy that can reduce local recurrence has been attempted, because, to date, there are no effective tools or modalities for drug delivery to the pancreatic bed. For the last several years our effort was focused on developing several trans-tissue, local delivery systems applicable to resected surface, using a photocured gelatin gel as tissue-adhesive matrices.[9–13] However, these systems do not have a drug reloading function. Herein, we developed a newly designed drug delivery device, which can be tightly attached to the resected surface, enables trans-tissue, local sustained drug delivery and reloading of the drug, and can be easily extracted.

GEM, an antimetabolite, exerts a citocidal effect on cancer cells in a time-dependent manner rather than in a dose-dependent manner. Several investigators reported that a long exposure to GEM markedly reduced IC_{50}.[21–24] However, adverse side effects due to prolonged systemic administration also appeared.[25–27] When 300 μg of GEM/body/week was continuously infused, all of the five mice lost weight considerably, and died (data not shown). On the other hand, when

less than 200 μg of GEM/body/week was continuously infused or when 1 mg of GEM was intraperitonealy injected, weight loss was not observed.

The device was solidly anchored on the target tissue using biodegradable sutures. As clearly shown in Figure 3, low-dose, local sustained GEM delivery using our present device significantly suppressed the growth of cancer tissue in heterotopic pancreatic cancer tumor model, and its regrowth after surgery was also suppressed. The difference in tumor growth characteristics between those of Groups III and V strongly indicates that local drug infusion near by or adjacent to tumor tissue is critical to effectively cure the tumor. The authors clearly envisage that an improved design and fabrication and optimized drug delivery conditions (dose, infusion speed, and period) can significantly increase the survival rate of patients who undergo surgery for pancreatic cancer. In addition, this device can find a versatile choice of infusible drug and versatile applications to other resected tissues such as liver tissue. Although the experiments in this study were very limited, it is highly envisaged that a promising applications with high therapeutic effectiveness and very low systemic side effect will be realized. Such experimental study leading to realize clinical application is now ongoing.

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Suppression of metastasis of human pancreatic cancer to the liver by transportal injection of recombinant adenoviral NK4 in nude mice

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NK4, a 4-kringle fragment of hepatocyte growth factor (HGF), is an HGF antagonist that also acts as an angiogenesis inhibitor. NK4 strongly inhibits the infiltration, metastasis, and tumor growth of pancreatic cancer. The aim of our study was to evaluate the antitumor effect of adenovirus-mediated NK4 gene transfer to the liver on hepatic metastasis of pancreatic cancer *in vivo*. We constructed recombinant adenoviral NK4 (Ad-NK4), which encodes a secreted form of human NK4. Intrasplenic injection of Ad-NK4 induced high and relatively maintained expression of NK4 protein in the liver and suppressed the number and growth of metastatic foci in the liver in a nude mouse model. Microscopically, central necrosis was found even in small metastatic foci in Ad-NK4 treated mice. Immunohistochemical analysis of metastatic tumors showed a remarkable decrease in microvessel density and an increase in the number of apoptotic tumor cells after treatment with Ad-NK4. These results indicate that intraportal injection of Ad-NK4 may be a useful therapeutic modality for the clinical control of hepatic metastasis in pancreatic cancer.

Key words: gene therapy; NK4; HGF antagonist; angiogenesis inhibitor; pancreatic cancer; hepatic metastasis; recombinant adenovirus

Pancreatic cancer remains one of the most malignant neoplasms. The disease is diagnosed frequently at an advanced stage, and only 3% of patients survive 5 years.[1] Pancreatic cancer has a high rate of local and systemic recurrence, including liver metastasis, peritoneal dissemination and retroperitoneal recurrence.[2] There is no effective treatment for this disease. Radical resection has only a limited effect for the disease,[3] but even after curative resection of the primary tumor, liver metastasis occurs frequently and constitutes a major course of this disease.[2,4] Generally, micrometastasis to the liver seems to have already occurred in most patients when pancreatic cancer is diagnosed.[5] Pancreatic cancer is highly resistant to the chemotherapy and radiation protocols available currently. Even gemcitabine, which has become the standard drug used for metastatic disease, does not improve median survival. One factor underlying the aggressive local and early systemic tumor growth may be rapid tumor neoangiogenesis, which results in an abundant blood supply. Tumor-induced neoangiogenesis is a common phenomenon during growth, particularly in tumors larger than 1–2 mm in diameter.[6,7]

We showed previously that pancreatic cancer cells frequently overexpress c-Met/hepatocyte growth factor (HGF) receptor and that HGF plays important roles in the mitogenic, motogenic and morphogenic activities of these cells. We also identified and prepared NK4 as an antagonist of HGF.[8,9]

NK4 is composed of the N-terminal hairpin and 4 kringle domains of HGF. NK4 binds c-Met/HGF receptor but does not induce tyrosine phosphorylation of c-Met. NK4 is a potent antiangiogenic agent and antagonizes not only HGF-induced angiogenesis but also that of other angiogenic factors such as vascular endothelial growth factor and basic fibroblast growth factor.[10] NK4 is also a competitive antagonist of HGF that completely inhibits HGF activity at concentrations of at least 1,000-fold the concentration of HGF.[11–13] Creation of an NK4-encoding adenovirus (Ad-NK4) has made it possible to produce sufficient concentrations of NK4 to antagonize the mitogenic, motogenic and morphogenic activities of HGF. *In vivo* studies, we showed that Ad-NK4 gene therapy inhibits the growth of pancreatic cancer in a subcutaneously (s.c.) implanted model and in an intraperitoneally (i.p.) implanted model.[14,15]

The adenovirus vector has higher specificity for the liver than for other organs[16–18] and can induce gene transfer in almost any cell type.[19] Intraportal injection of Ad-NK4 would be expected to result in high expression of NK4 in the liver, even at low infection concentrations.

We investigated the antitumor effect of intraportal injection of Ad-NK4 in a mouse model of pancreatic cancer metastasis to the liver.

Material and methods

Construction of recombinant adenovirus

A recombinant adenoviral vector expressing human NK4 (Ad-NK4) was constructed as described previously.[20] Briefly, Ad-NK4 was generated by homologous recombination of the pJM17 plasmid[21] and the shuttle plasmid vector pSV2+[22] containing an expression cassette and the cytomegalovirus early promoter/enhancer followed by human NK4 cDNA[11] and a polyadenylation signal. A control vector with expressing the bacterial β-galactosidase enzyme (LacZ) was constructed by the same procedure with pJM17 and pCA17 containing the LacZ gene. Recombinant Ad-NK4 and adenovirus LacZ (Ad-LacZ) were propagated in HEK-293 cells. The adenovirus titer in plaque-forming units (pfu) was determined by plaque formation assays with HEK-293 cells.

Cells and culture conditions

The SUIT-2 human pancreatic cancer cell line was generously provided by Dr. H. Iguchi (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.

Animals and intrasplenic implantation of pancreatic cancer cells

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. Suspensions of 2×10^6 SUIT-2 cells/0.1 ml were implanted by open injection into the spleen of nude mice under anesthesia.

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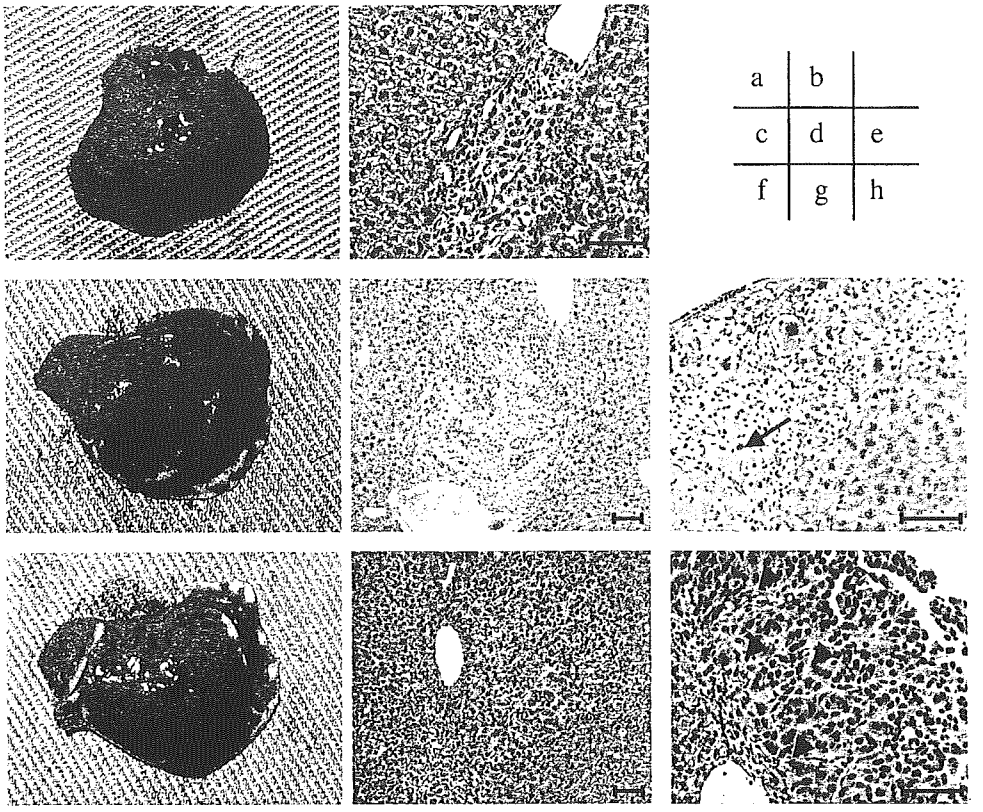

FIGURE 1 – Macroscopic and microscopic appearances of the liver on Day 3 after implantation of SUIT-2 cells (*a*,*b*), and of the liver in the Ad-NK4-treated group (*c*,*d*,*f*) and the PBS-treated group (*g*,*h*,*i*) on Day 7 after implantation of SUIT-2 cells. Scale bars = 10 μm. Long arrow, central necrosis; arrowheads, microvessels.

Treatment of intrasplenic implantation with Ad-NK4

Mice that received intrasplenic implantation of SUIT-2 cells were assigned to 1 of 3 treatment groups. Mice were re-laparotomised and then, Ad-LacZ or Ad-NK4 at 1×10^8 pfu/0.1 ml was injected into the spleen on Day 3 after intrasplenic implantation of SUIT-2 cells. In a control group, 0.1 ml PBS was injected in the same manner. Three mice from each group were killed on Day 3 and 3 more on Day 7 after intrasplenic implantation of SUIT-2 cells. Metastatic tumors were evaluated by histological examination. Five additional mice per group were killed on Day 21 after intrasplenic implantation and were evaluated with respect to the ratio of metastatic liver tumor volume to liver volume. Samples of metastatic liver tumors were also subjected to immunohistochemical study.

Determination of tumor–liver volume ratio

Livers were sectioned completely at 2-mm intervals. Images of all sections were digitized, and liver volume was determined with the use of NIH Image software. Tumor volume was determined in a similar manner.

NK4 expression in liver, lung, and blood serum after intrasplenic injection of Ad-NK4

To evaluate NK4 expression in abdominal tissues and blood serum, mice that had undergone intrasplenic injection of PBS, Ad-LacZ or Ad-NK4 were killed on Days 3, 7, 14, 21 and 28 after viral injection. Tissue samples of liver and lung were homogenized in ice-cold lysis buffer composed 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 after centrifugation at 12,000 rpm for 10 min. Expression of NK4 was detected with an ELISA kit (Immunis HGF EIA; Institute of Immunology, Tokyo Japan). The lower limit of detection of NK4 was 0.3 ng/1 g protein.

Immunohistochemical staining and cell counting

Livers from animals in each group were subjected to immunohistochemical staining on Day 21. Tissue sections (5-μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and rehydrated in graded alcohols. For microvessel staining, the peroxidase-conjugated avidin-biotin complex method was carried out with a Vectastain Elite ABC Kit (Vector, Burlingame, CA). Mouse monoclonal CD31 antibody JC/70A (Neo-Markers, Fremont, CA) was used at a dilution of 1:50, followed by incubation with biotinylated anti-mouse IgG (1:100; Vector). Microvessel density (MVD) was assessed in tumor areas showing high staining density. The number of vessels was counted in 10 fields per section at ×200 (0.739 mm^2/field), and the mean counts were recorded. Apoptotic cells within tumors were detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling TUNEL assay (*In situ* Apoptosis Detection Kit, Takara, Shiga, Japan). Positive cells were counted in each 10 randomly selected fields per section at ×200. Proliferating cells were detected with an antibody generated against proliferating cell nuclear antigen (PCNA) dilution PC10 (DAKO Glostrup, Denmark), and immunoreactivity was visualized in a manner similar to that used for CD31 staining. To quantify PCNA staining, we counted the positive cells in 10 random fields per section at ×200.

Toxicity

In tumor-free mice, Ad-NK4 at the same concentration as in previous experiments was injected into the spleen. On Days 3, 7, 14 and 21 after injection, mice were killed and blood samples were subjected to biochemistry tests such as GOT, GPT, glucose, BUN, creatinine and amylase activity. As a control, blood sera from mice with no treatment were also analyzed.

Survival

Mice were treated intrasplenic implantation of SUIT 2 cells and intrasplenic injection of PBS or Ad-NK4 in a similar manner as above. The condition of these mice was checked

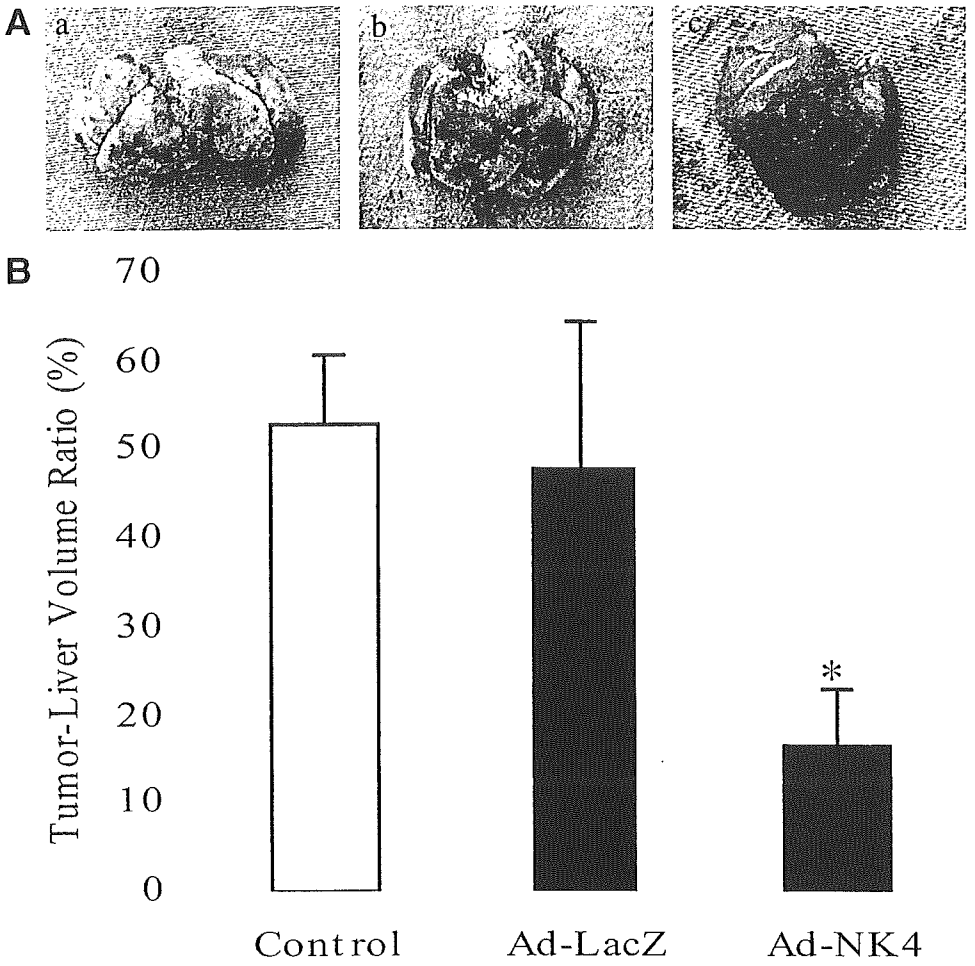

FIGURE 2 – Suppression of metastatic liver tumors by intrasplenic injection of Ad-NK4. PBS, Ad-LacZ, or Ad-NK4 was injected intrasplenically on Day 3 after implantation of SUIT-2 cells. (*a*) Macroscopic appearance of metastatic liver tumors after treatment with PBS (control; *a*), Ad-LacZ (*b*), and Ad-NK4 (*c*) on Day 21 after splenic implantation of SUIT-2 cells (n = 5/group). (*b*) Ratio of tumor volume *vs.* liver volume. Data are expressed as mean ± SD. *$p < 0.005$.

TABLE I – SUPPRESSION OF LIVER METASTASIS BY TRANSPORTAL INJECTION OF Ad-NK4 ON DAY 21 AFTER IMPLANTATION OF SUIT-2 HUMAN PANCREATIC CANCER CELL

	Liver weight (g)	Liver–body weight ratio (%)	Tumor–liver volume ratio (%)
PBS	2.49 ± 0.62	11.05 ± 2.82	52.75 ± 8.23
Ad-LacZ	2.23 ± 0.51	10.24 ± 2.46	48.39 ± 15.99
Ad-NK4	1.73 ± 0.28	7.46 ± 1.00	16.62 ± 6.60*

*Tumor–liver volume ratio was significantly lower in the Ad-NK4 treated group than other groups ($p < 0.005$).

out twice daily. Dead mice were autopsied as soon as possible at clean ventilator in facilities approved by Kyushu University.

Statistical analysis

Statistical significance was evaluated with a non-parametric Mann-Whitney *U*-test. All tests were 2-tailed, and a *p*-value of <0.05 was considered statistically significant. Survival was analyzed by log-rank analysis of Kaplan-Meier curves.

Results

Effect of Ad-NK4 on metastatic liver tumors

On Day 3 after intrasplenic implantation of SUIT-2 pancreatic cancer cells, there were few macroscopic metastatic nodules at the liver surface. Histologically, there were several micrometastatic nodules measuring approximately 30 μm in diameter (Fig. 1*a,b*). These were thought to be indicative of early liver metastases, which are not detectable by imaging. Therefore, we injected Ad-NK4 at this time. On Day 7 after implantation of SUIT-2 cells, no significant difference in macroscopic liver appearance between the PBS- and Ad-NK4-treated groups was identified (Fig. 1*c,f*). Histologically, metastatic tumors of the PBS-treated group showed severe invasion into the hepatic parenchyma, and neovascularization was already initiated (Fig. 1*d,e*). Metastatic tumors of the Ad-NK4-treated group showed expanding growth with pseudo capsules containing fibroblasts and inflammatory cells, but there was no neovascularization in the tumor and central necrosis was present (Fig. 1*g,h*).

Suppression of liver metastasis by intrasplenic injection of Ad-NK4

SUIT-2 cells exhibited an aggressive and malignant phenotype *in vivo*, and intrasplenically implanted cells never failed to produce metastatic nodules in the liver in the absence of Ad-NK4 injection. The macroscopic appearance of the liver showed almost complete coverage by metastatic tumors in both the PBS- and Ad-LacZ-treated groups. Intrasplenic injection of Ad-NK4 inhibited the growth of metastatic liver tumors remarkably compared to metastatic growth in the PBS- or Ad-LacZ-treated groups on

Day 21 after implantation (Fig. 2*a*). The respective tumor volume/liver volume ratio were 52.75 ± 8.23%, 48.39 ± 15.99% and 16.62 ± 6.60% for the PBS-, Ad-LacZ- and Ad-NK4-treated groups. There were no significant differences in liver weight between the groups (Table I, Fig. 2*b*).

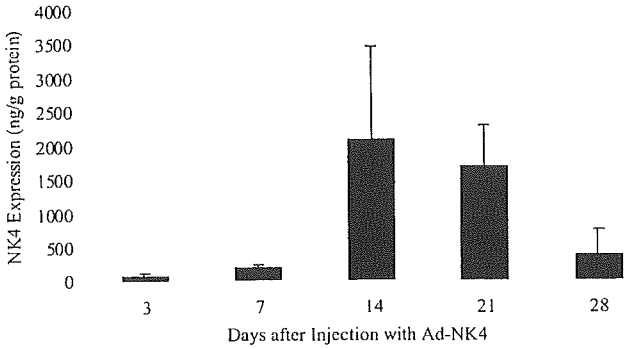

FIGURE 3 – Time-course of NK4 expression in livers after intrasplenic injection of Ad-NK4. Mice were killed 3, 7, 14, 21, and 28 days after intrasplenic injection of Ad-NK4. NK4 expression was detected by ELISA. Data are expressed as mean ± SD.

NK4 expression in liver, lung, and blood serum after intrasplenic injection of Ad-NK4

Intrasplenic injection of Ad-NK4 induced extremely high expression of NK4 in the liver (Fig. 3). NK4 expression was first identified on Day 3 (59.85 ± 61.87 ng/g protein), and it peaked on Day 14 after injection (2084.58 ± 1383.41 ng/g protein). The level remained high even on Day 28 after injection (366.62 ± 373.041 ng/g protein). In contrast, NK4 expression in the lung and blood serum was extremely low and was not detectable on Day 7 after injection. NK4 expression was not detected in other organs (data not shown). In the PBS- and the Ad-LacZ- group, NK4 expression was not detectable in all organs through every time points (data not shown).

Immunohistochemical examination of pancreatic tumors

Histologically, the pancreatic tumors showed moderate to poorly differentiated adenocarcinomas that were similar in all 3 leading index groups. With respect to PCNA staining, the difference between the 3 groups was not significant; the proliferation index was not altered by NK4 expression. The apoptotic index as determined by TUNEL staining was greater in the Ad-NK4-treated group (17.0 ± 1.7%; $p = 0.004$) than in the Ad-LacZ-treated group (8.7 ± 1.2%) or the PBS-treated group (9.7 ± 0.6%). MVD was significantly lower in the Ad-NK4-treated group (5.9 ± 1.4 vessels/field; $p < 0.001$) than in the Ad-LacZ-treated

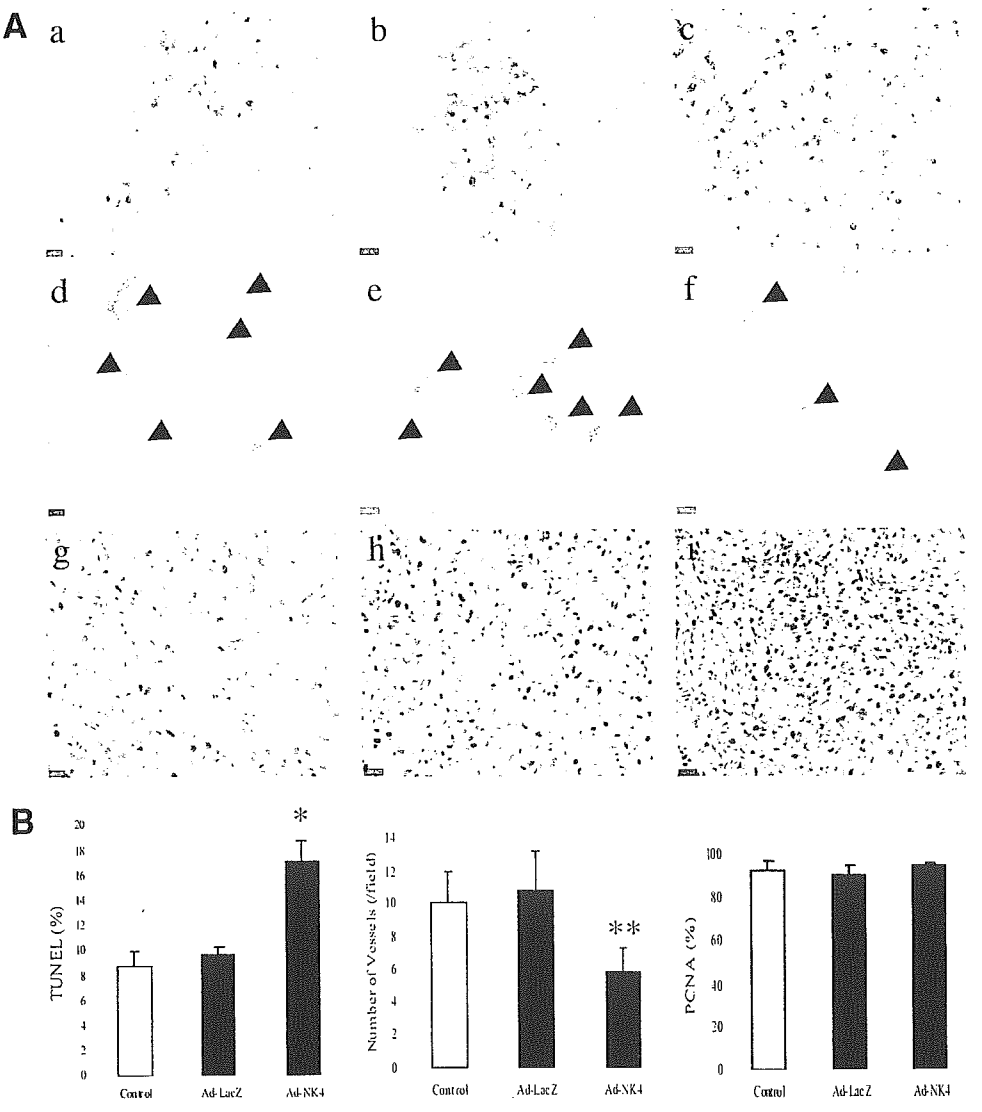

FIGURE 4 – Immunohistochemical staining of metastatic liver tumors on Day 21 after intrasplenic implantation of SUIT-2 cells, with intrasplenic injection of PBS, Ad-LacZ, or Ad-NK4. (*a*) Apoptosis (*a–c*), angiogenesis (*d–f*) and proliferation (*g–i*) were determined with the use of the TUNEL method, anti-CD31 antibody, and anti-PCNA antibody, respectively. PBS-treated group (*a,d,g*), Ad-LacZ-treated group (*b,e,h*), Ad-NK4-treated group (*c,f,i*). Arrows indicate microvessels. (*b*) Changes in the numbers of TUNEL-positive cells and blood vessels. Data are expressed as mean ± SD; $*p = 0.004$, $**p < 0.001$.

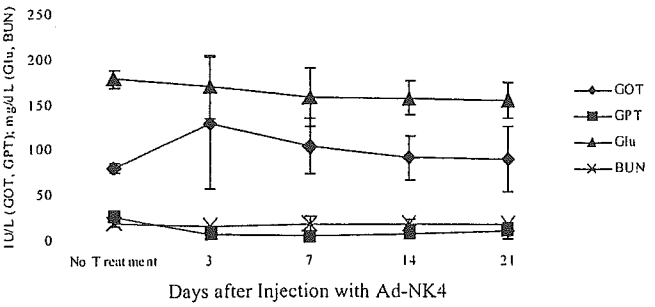

FIGURE 5 – Blood serum enzyme levels after intrasplenic injection of Ad-NK4. Mice were killed 3, 7, 14, and 21 days after intrasplenic injection of Ad-NK4. Data are expressed as mean ± SD.

group (10.8 ± 2.5 vessels/field) or in the PBS-treated group (10.1 ± 1.9 vessels/field) (Fig. 4*a*,*b*).

Toxicity of Ad-NK4

Blood biochemistry assays showed mild elevation of GOT on Day 3 after injection of Ad-NK4 that decreased rapidly to base line levels. Other enzymes showed no significant abnormalities (Fig. 5).

Survival

Almost all mice in our study died of cachexia or severe peritoneal dissemination. Survival times differed between the groups. Mice of the PBS-treated group become moribund starting at Day 19 after implantation, and the last mouse died on Day 36.The Ad-NK4-treated mice gradually died beginning at Day 19, but the last mouse died on Day 48. The median survival times of the PBS- and the Ad-NK4-treated group were 25.6 ± 7.3 and 39.0 ± 6.9 days, respectively (Fig. 6). The survival rate of the Ad-NK4-treated group was significantly greater than that of the PBS-treated group ($p = 0.01$).

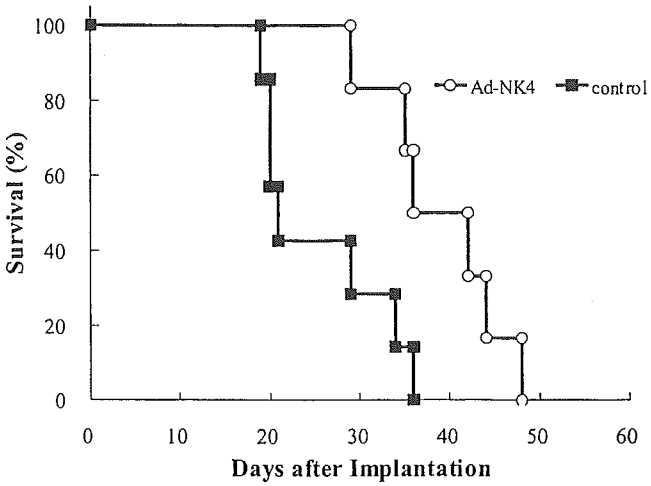

FIGURE 6 – Survival curves of PBS- ($n = 7$) and Ad-NK4-treated ($n = 6$) mice. PBS or Ad-NK4 was injected intrasplenically on Day 3 after implantation of SUIT-2 cells. There was a significant difference in survival times between the 2 groups ($p = 0.01$ by log-rank analysis of Kaplan-Meier curves).

Discussion

Our study showed that intrasplenic injection of Ad-NK4 significantly suppressed tumor progression of pancreatic cancer in the liver of nude mice and prolonged survival. A clinical correlate of our study would involve postoperative liver metastasis of pancreatic cancer without other clinical or radiologic evidences of the disease. NK4 expression in the liver in response to Ad-NK4 injection was sustained and high, and pancreatic tumors showed decreased numbers of tumor vessels and an increased tumor cell apoptotic index.

Many pancreatic cancer patients who undergo resection experience disease recurrence, most frequently in the liver[2,23] and usually within 1 year.[23] At the time of surgery, *K-ras* mutation in the liver has occurred in approximately 30% of patients, even when they showed no liver metastasis before surgery.[5] When pancreatic cancer is diagnosed, most patients may already have micrometastases in the liver. Therefore, it is vital to halt or limit liver metastasis, to inhibit enlargement of metastatic tumors and to inhibit spread to other parts of the liver or to other organs.

We reported previously that HGF may be involved in the aggressive invasion, dissemination or metastasis of postoperative pancreatic cancer and that NK4 can inhibit HGF-induced invasive and metastatic behaviors.[8,15,24] In our current study, we showed an antitumor effect of NK4 by intrasplenic injection of Ad-NK4 in a model of metastasis to the liver, suggesting the possibility that Ad-NK4 can inhibit metastatic liver tumors in postoperative pancreatic cancer patients.

The SUIT-2 human pancreatic cancer cell line overexpresses the c-Met receptor, and NK4 dose-dependently inhibits HGF-induced invasion and migration of these cells[8] and decreases the number of blood vessels surrounding the tumor.[14] Small tumors (1–2 mm in diameter) receive nutrients by diffusion, but when they grow to over 2 mm in diameter, they need an additional blood supply and therefore induce angiogenesis.[6,7] NK4 not only antagonizes HGF-induced angiogenesis but also arrests the angiogenesis induced by other angiogenic factors, such as vascular endothelial growth factor and basic fibroblast growth factor.[13] The bifunctional nature of NK4 may lead to its development as a key drug in tumor therapy.

NK4 is a competitive antagonist of HGF,[9,25] but NK4 binding to the c-Met receptor is 10-fold lower in affinity than HGF binding, and for complete inhibition of HGF binding to the c-Met receptor, 1,000-fold higher concentrations of NK4 over than that of HGF are required.[11–13] It is important to express as high a concentration as possible in the target organ. Use of the adenovirus vector is best for this purpose because it has higher specificity for the liver than for other organs[16–18] and can induce gene transfer in almost any cell type.[19] NK4 is a secretory protein, so it is not necessary for the vector to limit transduction to cancer cells. The adenovirus vector can show toxicity in the liver, muscle and lung when it is injected at high concentrations,[26] and it can also cause host immune responses when administered intravenously.[27] Use of minimal vector concentration to express maximum transfected gene product is necessary.

We administered Ad-NK4 to the spleen of mice as a model of intraportal injection. Expression of NK4 was very high in the liver and was prolonged over 28 days after administration; other organs expressed little or no NK4. Ad-NK4 induced minor and transient elevation of transaminase (GOT) levels. We attempted i.p. injection of Ad-NK4 at the same dose, but NK4 expression in the liver was very low in these experiments, and the suppression of tumor growth was disappointing (data not shown).

With respect to survival rate, significant increases were seen in Ad-NK4-treated mice and this was because that the tumor growth and dissemination from metastatic liver tumor were inhibited, so the onset of liver disfunction and intraabdominal bleeding were delayed, but all mice eventually died. One reason is that even though the adenovirus vector can induce high levels of transgene expression, this expression is temporary. We administered Ad-NK4 only once. In actual therapy, however, multiple injections of Ad-NK4 must be considered. Another reason is that NK4 shows

cytostatic effect; therefore the combination therapy with a cytocidal drug such as gemcitabine should enhance the anti-tumor effect.

Many studies have presented recently that various types of cancer such as gastric cancer, gallbladder cancer, glioblastoma and colon cancer overexpress c-Met/HGF receptor and NK4 strongly suppressed the tumor growth and invasion of these type carcinoma *in vivo*.[28–32] These data indicate that NK4 is certainly effective for various types of carcinoma.

In summary, we showed that intraportal injection of Ad-NK4 induced the expression of an effective concentration of NK4 in the liver and significantly suppressed liver metastasis in a nude mouse model. The survival of Ad-NK4-treated mice was significantly longer than that of PBS-treated mice. The therapeutic effect seemed to be due to the bifunctional activities of NK4 as an HGF antagonist and an angiogenesis inhibitor. Intraportal injection of Ad-NK4 may provide a benefit to patients with pancreatic cancer by inhibiting liver metastases after surgery.

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SURGERY TODAY

Onset of Liver Metastasis After Histologically Curative Resection of Pancreatic Cancer

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Abstract

Purpose. We assessed the possibility of predicting the time of onset of liver metastases by measuring the postoperative changes in serum carbohydrate antigen (CA)19-9 after curative resection of pancreatic cancers.

Methods. Among 28 patients who underwent histologically defined curative resection of pancreatic cancer between 1984 and 1999, liver metastasis developed in 11 patients with elevated serum CA19-9 levels. We plotted the serum CA19-9 levels against time on a semilogarithmic graph. Over the linear part of the curve, the time when log[CA19-9] equaled zero was defined as the time of onset of liver metastases. The log[CA19-9] level doubling time was then calculated and evaluated in relation to the survival period.

Results. The serum CA19-9 levels increased linearly in 10 of the 11 patients. The predicted time of onset of liver metastasis ranged from preoperative day 163.0 to postoperative day 27.1, being preoperative in eight patients. The doubling time until death correlated strongly with survival in the eight patients with maintained log[CA19-9] linearity.

Conclusion. The onset of liver metastases might be preoperative in patients with advanced pancreatic cancer. Therefore, neoadjuvant chemotherapy should be mandatory even if there is no sign of liver metastases.

Key words Pancreatic cancer · Tumor marker · Carbohydrate antigen 19-9 · Doubling time · Liver metastasis

Introduction

The results of treatment for pancreatic cancer are extremely poor, despite intensive efforts. We use a combination of extended radical pancreatectomy and intraoperative radiation therapy to achieve histologically defined curative resection for pancreatic cancer, and prevent local recurrence.[1,2] Although this treatment helps to control local recurrence, survival has not improved dramatically because of the frequency of deaths associated with hematogenous metastases, especially in the liver. Almost all of these patients die within 2 years of surgery. Although liver metastases might exist before resection, in the form of latent lesions, to our knowledge there is no report providing evidence of this.

To improve the results of treatment for pancreatic cancer, we need more detailed information on the development of liver metastases. As yet, there is no reliable marker of liver metastases; thus, we investigated whether postoperative changes in carbohydrate antigen (CA)19-9 levels correlated with the predicted time of onset of liver metastases, and with prognosis.

Patients and Methods

Between 1984 and 1999, 41 patients with pancreatic cancer underwent a combination of extended resection and lymphadenectomy plus intraoperative radiation at Kumamoto University Hospital. The extended operation for pancreatic cancer consisted of pancreatectomy with almost complete dissection of the lymph nodes around the porta hepatis, the celiac axis, the mesenteric radix, and the aorta, from the level of the diaphragm down to the inferior mesenteric artery. After the tumor was resected, intraoperative radiation was delivered to an area of 6 × 10–14 cm, including the tumor bed, to a dose of 30 Gy with 8–12 MeV of an electron beam.[4]

Among 28 patients who underwent histologically confirmed resection of pancreatic cancer, 13 died of liver metastases, 11 of whom had positive serum CA19-9 levels. We retrospectively analyzed these 11 patients,

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Table 1. Clinical characteristics of the 11 patients

Patient No.	Age (years)	Sex	Stage	Preoperative CA19-9
1	48	M	II B	354.0
2	70	M	II B	72.1
3	40	F	IV	110.0
4	68	M	IV	1006.0
5	60	M	II B	85.0
6	70	F	II A	488.2
7	62	M	II B	1083.8
8	44	M	II B	2226.2
9	65	F	I A	51.7
10	73	F	II B	4700.0
11	71	F	II A	426.7

Mean age: 61.0 ± 3.3 years; M:F = 6:5
CA, carbohydrate antigen

who had no sign of local recurrence on postoperative computed tomography (CT) scanning or ultrasonographic examination. Their mean age was 61.0 years (range, 40–73 years), and there were six men and five women. The cancer stage at the time of resection was p-Stage IA in one patient, p-Stage IIA in two, p-Stage IIB in six (54.5%), and p-Stage IV in two. Although both of the patients with p-Stage IV cancer had metastases in the lymph nodes around the aorta, these were resected and the operation was judged to be curative. The mean preoperative serum CA19-9 level in the 11 patients was 964.0 (range, 51.7–4700) (Table 1).

We plotted the postoperative changes in CA19-9 levels on a semilogarithmic scale. The time when log[CA19-9] equaled zero was defined as the time of onset of liver metastases, on the linear part of the curve. The influence of the local lesion was excluded if follow-up CT scans showed no sign of local recurrence after curative resection. We judged that the change in log[CA19-9] reflected the status of liver metastasis exactly. Thus, the zero point on this line represented the time of onset of the liver metastases. Furthermore, if liver metastases were the cause of death, the number of days it took for the log[CA19-9] levels to double within the period of exponential increase was defined as the doubling time. We then investigated the correlation between the prognosis and the doubling time.

Hepatic metastases were evaluated preoperatively by CT scanning and ultrasonography, intraoperatively by ultrasonography, and postoperatively every 3 months by CT scanning and ultrasonography. Serum CA19-9 levels were also measured once a month postoperatively.

Statistical analysis was performed with a statistical software program (Statview; Abacus Concepts, Berkeley, CA, USA). Fisher's exact test and Wilcoxon's test were used for statistical evaluation with $P < 0.05$ considered significant. All results are expressed as mean ± SEM.

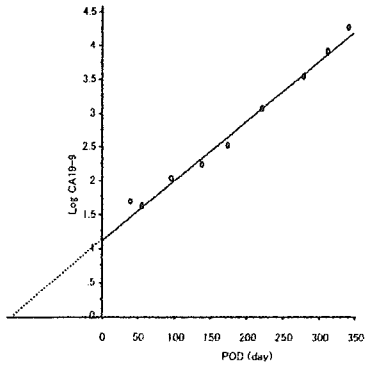
Results

Prediction of the Time of Onset of Liver Metastases

The actual 5-year survival rate of the 28 patients who underwent curative resection was 21.8%. Thirteen (65.0%) of the 20 deaths were caused by liver metastases, but 2 of these 13 patients were excluded from the analysis because their preoperative CA19-9 levels were negative. The CA19-9 levels decreased in the remaining 11 patients, but returned to within the normal range 1–2 months postoperatively in only 5. Ten patients had a linear increase in the postoperative log[CA19-9], which correlated well with the postoperative time (Fig. 1). The postoperative CA19-9 level did not increase in the other patient after liver metastases appeared. The predicted time of onset of liver metastases in ten patients ranged from preoperative day 163.0 to postoperative day 27.1, with preoperative metastases being confirmed in eight patients (mean preoperative day 62.7) (Table 2).

Prediction of Prognosis and Survival Based on Doubling Time

In the eight patients with a constant linear relationship throughout the observation period, postoperative survival and the doubling time were well correlated (Fig. 2). The mean CA19-9 doubling time was 20.8 days in the eight patients who died of liver metastases (Table 3). One of the remaining two patients suffered postoperative complications, which may have affected their survival. The other patient showed a biphasic change in the CA19-9 level, which increased rapidly during the postoperative course. Therefore, the doubling time was calculated twice from the plotted line of this patient, who died of blood-borne metastases. The doubling time increased from 150.5 to 301.0 within about 1 year after the operation. We think that tumors with different grades



Patient No.	R (Coefficient of correlation)	p
No. 1	0.964	<0.0001
No. 2	0.969	0.0006
No. 3	0.911	<0.0001
No. 4	0.969	0.0054
No. 5	0.973	0.0041
No. 6	0.999	<0.0001
No. 7	0.999	<0.0001
No. 8	0.973	<0.0001
No. 9	0.999	<0.0001
No.10	0.962	<0.0001
No.11	0.09	0.535

Fig. 1. Postoperative change in log[CA19-9] levels showed a linear increase in 10 of 11 patients who underwent curative resection of pancreatic cancer. The zero point of the log[CA19-9] was considered the day of onset of liver metastasis. *POD*, postoperative day

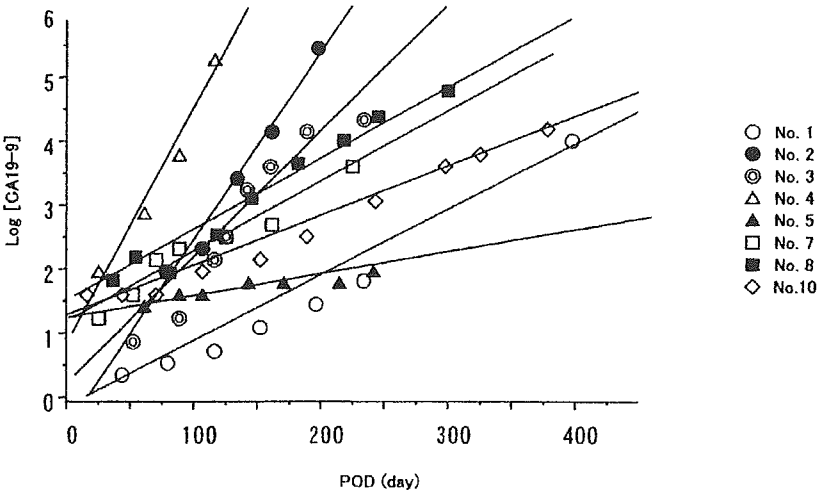


Fig. 2. Postoperative changes in the log[CA19-9] level showed a constant increase in 8 of 10 patients during the observation period. *POD*, postoperative day

Table 2. Predicted time of onset of liver metastasis in the 10 patients

Patient no.	Predicted time of CA19-9 levels at zero (operation day = 0)
1	27.1
2	19.8
3	−13.0
4	−30.5
5	−45.7
6	−58.4
7	−106.8
8	−121.6
9	−134.0
10	−163.4
Average	−62.7

of malignancy gained power while repeating the division.

The correlation coefficient between the survival period and the CA19-9 doubling time was 0.877, with a P value of 0.0023 in these eight patients (Fig. 3). Patient no. 5 underwent transcatheter arterial embolization (TAE) and patient no. 8 received a transarterial infusion of anticancer drugs for the metastatic liver lesions. However, the linearity of the CA19-9 curve during the observation period was hardly affected. None of the other patients received adjuvant chemotherapy. All liver metastatic nodules appeared fully developed on CT scans. When the survival time was replaced with the length of time after onset of liver metastasis, the correlation coefficient was 0.906, with a P value of 0.0008, yielding an even higher correlation (Fig. 3).

Discussion

To control local recurrence of pancreatic cancer, we routinely performed a combination of extended radical pancreatectomy with intraoperative radiation therapy.[1] Unfortunately, this approach has failed to achieve great improvement in the treatment results. We examined the factors affecting prognosis by conducting autopsies,

Table 3. Log[CA19-9] doubling times and survival periods in the 8 patients

Patient No.	Doubling time (days)	Survival period (months)
1	27.4	16.0
2	15.1	7.6
3	10.4	6.9
4	8.4	6.0
5	20.1	9.0[a]
7	20.1	11.3
8	27.4	17.5[b]
10	37.6	15.0
Average	20.8 ± 9.75	11.2 ± 4.5

[a]Transarterial embolization
[b]Transarterial infusion

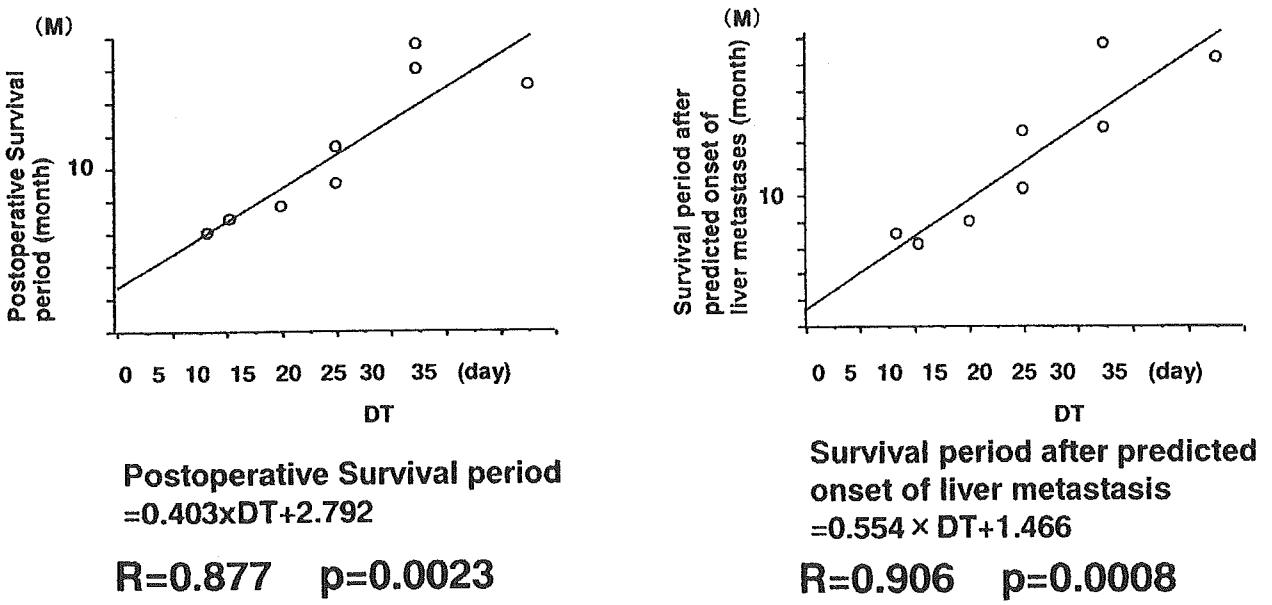


Fig. 3. We examined the relationship between the log[CA19-9] doubling time (*DT*) and the survival period. The survival period after surgery and that after the onset of liver metastases were both significantly related to the doubling time

which revealed that although the treatment effectively prevented local recurrence, blood-borne metastases, especially to the liver, greatly affected the overall prognosis.[2]

To improve the results of treatment for pancreatic cancer, effective measures against blood-borne metastases, especially liver metastases, are urgently required. However, few studies have been done on the time of onset of liver metastases in relation to surgery. It has been suggested that the liver metastases emerging postoperatively originate from undetected liver micrometastases,[3] intraoperative dissemination of cancer cells, and local recurring lesions. Safi et al. reported that CA19-9 measurement is a simple test, which can be used for diagnosis as well as for predicting resectability, survival after surgery, and the potential for recurrence.[4] Montgomery et al. reported that measuring postoperative levels of CA19-9 was the best predictor of disease-free survival and median survival.[5]

Staab et al. examined time-related changes in levels of carcinoembryonic antigen, another tumor marker, in patients with gastrointestinal tract cancer, and analyzed the relationship between those changes and prognosis.[6] Takahashi et al. reported that changes in tumor markers correlated strongly with an increase in tumor size, representing an exponential increase, and demonstrated a correlation between doubling time and survival periods.[7] Although some reports suggest that changes in CA19-9 levels were useful for predicting the prognosis of patients with pancreatic cancer,[8-11] or as a

marker of hepatic metastases,[12,13] the usefulness of monitoring changes in CA19-9 levels based on theoretical calculations has not been analyzed. Our findings reinforce the need for liver metastases. The CA19-9 levels were negative in 2 of our 13 patients, both of whom died of liver metastasis within 2 years, indicating a high possibility that liver metastasis had already existed before the operation. Even if CA19-9 is negative in the early postoperative period in patients with advanced cancer, recurrence is likely, so regular postoperative examination is mandatory.

There is little doubt that the rate of tumor growth is a prognostic factor. In the present study, we found that the doubling time of the serum CA19-9 levels was closely correlated with the postoperative survival period, and even more closely correlated with survival after the onset of liver metastases. These results suggest that liver metastases greatly affect the prognosis of pancreatic cancer; thus, we must establish measures to prevent metastasis. Even if an advanced pancreatic cancer is resectable, occult liver metastases may already exist at the time of surgery and neoadjuvant chemotherapy is necessary to prevent postoperative hepatic recurrence. Otherwise, the existence of micrometastasis would contraindicate surgery.

In conclusion, the onset of liver metastases might be preoperative in patients with advanced resectable pancreatic cancer. Thus, we must find effective neoadjuvant chemotherapy to prevent liver metastases. Otherwise, these patients should be excluded from resection by preoperative selection. These important issues need to be resolved in the search for more effective treatment against pancreatic cancer.

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5-Fluorouracil Intra-arterial Infusion Combined With Systemic Gemcitabine for Unresectable Pancreatic Cancer

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Objectives: The aim of this study was to define assessment of response and adverse events of the combination chemotherapy of 5-fluorouracil (5-FU) pancreatic and hepatic arterial continuous infusion and systemic gemcitabine administration for unresectable pancreatic cancer.

Methods: We treated 24 chemotherapy-naive patients with unresectable pancreatic cancer. To prevent gastroduodenal injury from 5-FU infusion, the catheter was placed to allow the distribution of 5-FU to the pancreatic tumor and the liver after occlusion of the gastric and pancreaticoduodenal arteries. 5-FU was administered at a dose of 250 mg/d on days 1 to 5 every week as a continuous arterial infusion. Gemcitabine was infused intravenously at a dose of 1000 mg once weekly for 3 consecutive weeks of every 4 weeks.

Results: The partial response rate was 20.8% (5 of 24), although there was no case of complete response. Fourteen cases (58.3%) were stable disease, and 5 cases (20.8%) were progressive disease. The most common toxicities were hematological and gastrointestinal events. No patients died of adverse effects using this chemotherapy. Gastric and/or duodenal ulcers occurred because of 5-FU intra-arterial infusion. Catheter-related cholangitis occurred in patients with biliary drainage for obstructive jaundice. Median survival time was 14 months, with a 50.9% 1-year survival rate, although patients with performance status 2 and multiple organ metastases had a poor prognosis.

Conclusions: This combination chemotherapy was well tolerated and seemed to be effective for patients with unresectable pancreatic cancer.

Key Words: 5-fluorouracil intra-arterial infusion, distant metastases, unresectable pancreatic cancer

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Cancer of the pancreas has increased in incidence over the past several decades.[1] Despite improvements in imaging technology, less than 20% cases are potentially resectable at the time of initial diagnosis.[2] Unfortunately, most patients have locally advanced or metastatic diseases. For advanced cases, the 5-year survival rate is less than 1%, with most patients dying within 1 year of diagnosis. Gemcitabine has served as the standard of chemotherapy, based on its clinical benefit and improved survival in a phase 3 trial.[3] Moreover, the action of gemcitabine seems to be synergetic with 5-fluorouracil (5-FU).[4] In addition to this fact, the intra-arterial infusion of antineoplastic agents gives higher concentrations to the targeted part compared with whole body administration and is associated with lower toxicity.

The primary endpoint of this study was the objective response rate. Secondary endpoints studied included characterization of the toxicity profile and median survival.

PATIENTS AND METHODS

Patients Selection

Between January 2001 and January 2004, we treated 24 chemotherapy-naive patients with unresectable pancreatic cancer. Locally advanced or metastatic adenocarcinoma of the pancreas was histologically or cytologically confirmed in 11 patients. To be included in this study, patients had to have an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤ 2, including the following: absolute neutrophil count $\geq 1500/mm^3$, bilirubin ≥ 1.5 mg/dL, aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels ≤ 3 times the upper limit of normal, and creatinine ≤ 2 mg/dL. All patients were advised of the investigational nature of the study and gave written informed consent to participate before the study.

Catheter Implantation

A 5-Fr catheter was inserted from the femoral artery with the Seldinger technique. The right and left gastric arteries and the anterior and posterior superior pancreaticoduodenal arteries were occluded by microcoils to prevent gastroduodenal injury from 5-FU infusion and to simplify blood flow to the pancreas and liver. Under local anesthesia, the catheter for arterial infusion was introduced from the branch of the left subclavian artery in 14 cases and from the femoral artery in 10 cases. After the closure of the distal tip of the catheter, a side hole was made at an appropriate site in the celiac axis to allow

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the distribution of 5-FU to both the pancreatic tumor and the whole liver. An arterial port was implanted in the subcutaneous tissue.

Drug Administration

Gemcitabine diluted in normal saline was infused intravenously for 30 minutes at a dose of 1000 mg once weekly for 3 consecutive weeks of every 4 weeks. 5-FU was administered at a dose of 250 mg/d on days 1 to 5 every week as continuous infusion through the arterial port. One cycle length was defined as 4 weeks. In case of grade 2 or more toxicity according to National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 2.0, drug infusion was interrupted until recovery. Patients were allowed to remain on treatment providing there was no evidence of progressive disease and/or PS $\geq$3. Complete blood counts (CBCs) were repeated weekly before infusion of the drugs. Chemistry profiles were performed every month.

Pretreatment and Follow-up Evaluations and Assessment of Response Rate

A history and physical examination were performed before this study and before each infusion. Studies included CBC, serum AST, ALP, total bilirubin, creatinine, sodium, potassium, and glucose. Computed tomography for response evaluation was performed every 4 weeks. The therapeutic responses were evaluated according to World Health Organization criteria. A complete response (CR) was defined as the complete disappearance of all assessable disease for at least 4 weeks. A partial response (PR) was defined as a decrease of at least 50% in the sum of the products of the bidimensional diameters of measurable lesions for at least 4 weeks. Stable disease (SD) was defined as the decrease of less than 50% or the increase of less than 25% in tumor size. Progressive disease (PD) was defined as an increase of at least 25% or appearance of new neoplastic lesions.

Toxicity and Survival Evaluation

This study used the NCI-CTC version 2.0 adverse event monitoring and reporting. Toxicity was evaluated before each cycle of treatment. The maximum grade for each type of toxicity was recorded.

In all patients, the date of initial treatment was chosen as the starting point for survival analysis. Overall survival was determined from day 1 of treatment until death. The survival curve was drawn with the Kaplan-Meier method.

RESULTS

Patients Characteristics

The characteristics of the 24 patients are outlined in Table 1. Sixteen of the patients were men and 8 were women, with a median age of 62.6 years (range, 42–76 years). The primary pancreatic lesion was located in the head in 12 patients, in the body in 9 patients, and in the tail in 3 patients. Of the 24 patients, 21 patients (87.5%) had distant metastases. The most common site of metastases was the liver (17 patients). Three patients suffered from multiple organ metastases. Nine patients had a PS of 0. Ten patients had a PS of 1.

TABLE 1. Patients Characteristics

No. of patients	24
Age (yr)	
Median	62.6
Range	42–76
Male/female	16/8
ECOG performance status	
0	9
1	10
2	5
Site of primary lesion	
Head	12
Body	9
Tail	3
Site of metastases	
None	3
Liver	17
Dissemination	1
Multiple	3

Five patients had a PS of 2. Patients received a minimum of 3 cycles and a maximum of 12 cycles.

Toxicity

Hematological and nonhematological toxicities related to therapy are outlined in Table 2. No potentially life-threatening toxicity was seen. The most common toxicities were hematological and gastrointestinal events. All patients had leukopenia, which abated after discontinuation of drug infusion. One patient developed grade 4 neutropenia, which also abated after interruption of drug infusion. Eighteen patients had anemia during treatment. Two of 3 patients with grade 3 anemia required blood transfusion. Thrombocytopenia was mild and occurred in 8 patients (30%). Toxicities distinctively related to 5-FU arterial infusion were gastric and/or duodenal ulcer (4 patients). Gastric and/or duodenal ulcer was healed by treatment with antiulcer agents and discontinuation of 5-FU intra-arterial infusion. Catheter-related

TABLE 2. Treatment-related Toxicities for This Combination Therapy

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Hematological				
Anemia	8	6	4	0
Leukopenia	5	13	6	0
Neutropenia	4	7	7	1
Thrombocytopenia	6	2	0	0
Gastrointestinal				
Nausea	9	6	0	0
Gastric/duodenal ulcer	0	4	0	0
Others				
Cholangitis	0	2	3	0
Cerebral infarction	1	0	0	0

Values are numbers of patients.

cholangitis occurred in 5 patients who had biliary drainage for obstructive jaundice and were treated by appropriate exchange of the drainage tube. One patient experienced mild cerebral infarction with partial defect of the visual field. Another patient experienced dislocation of the infusion catheter, which required reimplantation of the catheter.

Therapeutic Response and Outcome

The PR rate was 20.8% (5 of 24), although there was no case of CR. Fourteen cases (58.3%) were SD, and 5 cases (20.8%) were PD.

The overall survival curve is depicted using the Kaplan-Meier method in Figure 1. The median overall survival was 14 months. Survival at 6, 12, and 24 months was 83.3%, 50.9%, and 12.7%, respectively. Five patients, who obtained PR during the follow-up period, had been alive more than 15.3 months at the time of data analysis (range, 15.3–25 months). Patients with a PS of 0 to 1 have survived significantly longer than patients with a PS of 2 (Fig. 2). Patients with a PS of 2 had a poor survival (4.5–7.9 months). All patients without distant metastases were alive more than 1 year. On the other hand, all patients with multiple distant metastases died within 1 year (range, 4.5–12 months; Fig. 3). No patients died as a result of adverse effects of chemotherapy. PS did not change during treatment until the progression of diseases.

DISCUSSION

The primary endpoint of this trial was the objective response with secondary endpoints of safety and median survival. The expected goal was to identify the efficacy of this combination chemotherapy for survival benefit for unresectable pancreatic cancer.

The response rate was 20.8% in our study, which is compatible with that in another trial with the same combination chemotherapy in patients with pancreatic and biliary tract cancer (25%).[5] Gemcitabine has shown antitumor activity in patients with carcinoma of the pancreas and is widely used as first-line chemotherapy in the treatment of this disease. However, despite superior activity compared with 5-FU, the result achieved with single-agent gemcitabine in pancreatic cancer is still poor (reported response rate of 5.4%, a median survival time of 5.65 months, and a 1-year survival rate of 18%).[3] It is thus indicated that the addition of 5-FU intra-arterial infusion to systemic gemcitabine might have efficacy compared with gemcitabine alone. To our knowledge, other combinations of intra-arterial and systemic chemotherapy have not been reported in patients with unresectable pancreatic cancer.

The rationale for intra-arterial infusion of chemotherapeutic agents seems to be promising from the point of view of the drug's concentration-response, because most liver metastases (>3 mm) have an arterial blood supply.[6,7] Moreover, intra-arterial infusion is considered to take advantage of the first pass effect of the drug, generating higher local drug concentrations at tumor cells with a lower toxicity. Continuous intra-arterial infusion may be advantageous in maintaining the

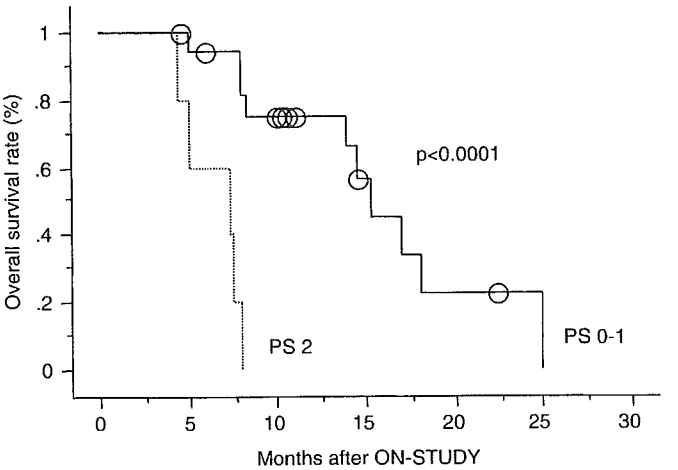

FIGURE 2. Kaplan-Meier survival curves for patients with PS 0-1 or PS 2. $P < 0.001$ (log-rank test).

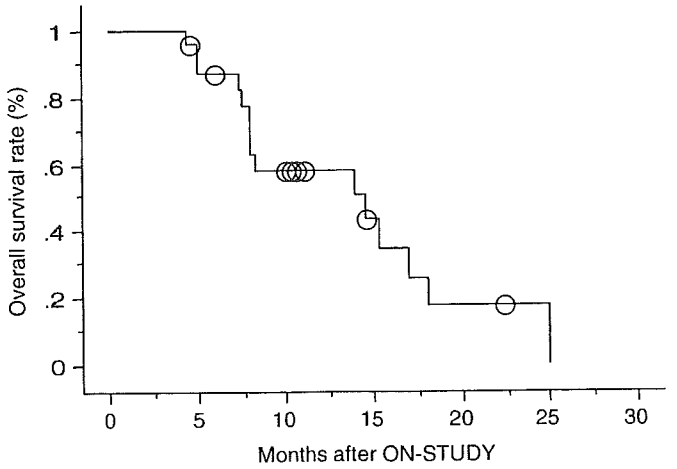

FIGURE 1. Overall survival for patients receiving 5-FU intra-arterial infusion and systemic gemcitabine for unresectable pancreatic cancer.

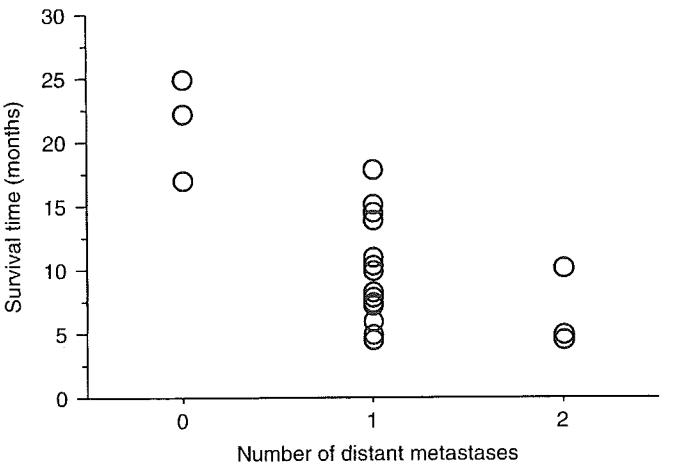

FIGURE 3. Relationship between survival time and number of distant metastases.

concentration of time-dependent chemotherapeutic agents such as 5-FU. On the other hand, systemic blood concentration of 5-FU may be insufficient to treat occult extrahepatic metastases, because 5-FU has a high hepatic first-pass effect of up to 50%.[8] Although no evidence of an advantage in progression-free survival or overall survival of the intra-arterial infusion compared with systemic therapy was reported for colorectal liver metastases,[9] a significantly higher response rate has been reported.[10] Pancreatic and hepatic arterial infusion chemotherapy for pancreatic cancer may not be enough, because extraabdominal metastases were documented in 27% of patients, even after curative resection of pancreatic carcinoma.[11] Therefore, we believe systemic chemotherapy with gemcitabine combined with regional and hepatic intra-arterial infusion may be necessary for the treatment of pancreatic cancer.

This combination chemotherapy was well tolerated in all patients. Patients could receive a maximum of 12 cycles. There were no potentially life-threatening adverse events. Most toxicities were mild hematological events. Patients experienced grade 3 or 4 anemia (16.7%), leucopenia (25.0%), and neutropenia (33.3%). One adverse effect of 5-FU intra-arterial infusion was gastric and/or duodenal ulcers. Before introducing coil embolization of gastric and duodenal arterial branches, we previously experienced duodenal obstruction caused by duodenal edema or gastric inflammation. Although duodenal obstruction did not occur in this study, gastric and/or duodenal ulcer still occurred. Prevention of gastroduodenal injury from 5-FU infusion might be difficult even after coil embolization of gastric and duodenal arterial branches. Cholangitis is an adverse effect specific to patients with obstructive jaundice. Special attention needs to be paid to patients with obstructive jaundice, because chemical cholangitis caused by 5-FU infusion aggravates inflammation. In 4580 cases of hepatic artery infusion by Barnett et al,[12] the most common toxicities included gastrointestinal symptoms in 22%, chemical hepatitis in 19%, and bone marrow toxicities in 8%. Catheter implantation was radiologically performed in this study. Another problem associated with intra-arterial infusion chemotherapy is catheter-associated complications. According to Heinrich et al,[13] catheter-associated complications occurred in 4% to 56% of cases. In this trial, 1 patient (4.2%) suffered from mild cerebral infarction as a catheter-related complication.

This trial showed that the combination of regional 5-FU intra-arterial infusion and systemic gemcitabine was effective for unresectable pancreatic cancer, with a median survival time of 14 months and a 1-year survival rate of 50.9%. However, patients with a PS of 2 had poor survival. Moreover, patients with multiple distant metastases also died within 1 year. Therefore, this combination chemotherapy may not be indicated for patients with a PS of 2 and/or multiple distant metastases.

There are several limitations in this study. This was a nonrandomized study with a small series of 24 patients. Adenocarcinoma was pathologically confirmed in 11 patients. Moreover, we adopted "milligrams per body" instead of "milligrams per square meter" for doses of these drugs. Because the study was a preliminary one, we mainly focused on feasibility of the clinical protocol. However, it is necessary to plan a randomized control study to clarify the efficacy of this combination chemotherapy for survival benefit against only systemic chemotherapy in the future. Doses of drugs should be determined in proportion to body dimensions. Pathologic confirmation in all patients is required before enrollment onto a clinical study.

In conclusion, this combination chemotherapy was well tolerated and seemed to be effective for patients with unresectable pancreatic cancer. Moreover, this combination chemotherapy should be assessed as an adjuvant chemotherapy after curative operation to improve survival.

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Identification of Prognostic Factors Associated with Early Mortality after Surgical Resection for Pancreatic Cancer—Under-analysis of Cumulative Survival Curve

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Abstract

Background: The cumulative survival curve after surgery for advanced pancreatic cancer is characterized by a steep downward slope in the early postoperative period. The aim of this investigation was to identify the characteristics associated with early mortality in patients undergoing pancreatic resection for pancreatic cancer.

Methods: Thirty-seven patients with extended radical pancreatectomy combined with intraoperative radiation therapy were studied. The cumulative survival curve in this series was depicted using the Kaplan-Meier method. Assuming that there were two distinct survival curves, below and above the breakpoint, each part of the curve was modeled as an exponential distribution. Three parameters, the breakpoint, the high hazard rate below the breakpoint, and the low hazard rate above the breakpoint were estimated by the maximum likelihood method. Prognostic factors associated with early mortality after surgery were evaluated using univariate and multivariate Cox proportional hazards regression analyses.

Results: The breakpoint of the survival curve was estimated at 41 months. The short-survival group (SSG) was defined as deceased earlier than 41 months after surgery, and included 31 patients (83.8 %). The long-survival patient group (LSG) consisted of 6 patients who were alive more than 41 months after surgery. Eighteen SSG patients (58.1 %) died of hepatic metastases, whereas no LSG patients died of hepatic metastases. Abdominal pain and/or back pain during clinical course was identified by multivariate analysis as a prognostic factor for patients undergoing pancreatic resection.

Conclusions: The high hazard rate in the early postoperative period was closely linked with death due to liver metastases. The preoperative presence of local pain was a prognostic factor associated with early mortality.

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Pancreatic adenocarcinoma remains a lethal disease. Despite improvements in imaging technology regarding diagnosis, fewer than 20% of cases have been potentially resectable at the time of initial diagnosis.[1]