using a combination of HART (three fractions of 1.1 Gy, total 45–50 Gy) and continuous infusion of 5-FU (25 mg/kg/day) the first and third week. They treated 13 patients, and the local response rate and the MST were 23.1% and 36 weeks, respectively. More recently, Ashamalla et al. (13) and Ueno et al. (14) conducted HRT using relatively high dose of irradiation (two fractions of 1.1 Gy delivering a total 63.8 Gy and two fractions of 1.2 Gy delivering a total 45.6–64.8 Gy, respectively) combined with chemotherapy during RT course. Treatment duration was 4–6 weeks. The local response rate was 29.4 and 20.7%, respectively. The MST was 10 and 12.2 months, respectively, and the 1-year survival rate was 45.0 and 55.0%, respectively.

In our Phase I study, the final planned dose (45 Gy) did not exceed the MTD as defined in the protocol. Therefore, patients received 45 Gy irradiation in the subsequent Phase II study. Six patients in the Phase I study and an additional 14 patients enrolled in the Phase II study received recommended dose of irradiation, and a total of 20 patients were evaluated for the local efficacy and survival: these patients consisted of 10 patients with locally advanced lesions (Stage 4A) and 10 patients with metastatic lesions (Stage 4B). The MST and the overall 1-year survival rate of all 20 patients were 11.2 months and 40.0%, respectively. The outcome of Stage 4A patients was relatively good: the MST and 1-year survival rate were 13.0 months and 70.0%, respectively, although the total dose of RT used in this study was almost equal or lower than that used in other HRT of HART trials (Table 5). Moreover, concerning primary tumors, 7 patients (35%) showed PR, 10 (50%) remained SD, and local PD was observed in only 3 (15%) patients. These findings encouraged us to perform further trials of HART-based CRT for Stage 4A patients. On the other hand, the results of Stage 4B patients were dismal. All patients diagnosed as Stage 4B had liver metastasis, and they all received additional treatment of continuous hepatic arterial infusion of 5-FU. The chosen treatment, however, did not improve the survival in patients with liver metastasis: the MST and 1-year survival rate were 5.0 months and 10.0%, respectively. Therefore, more effective treatment modalities are needed to control liver metastasis.

In our treatment schedule, patients could complete the treatment with a shorten time of only 3 weeks: this duration is 40% shorter than that of conventional treatments usually ranging over 5 weeks. The overall treatment time was important because the life expectancy of patients with advanced pancreatic cancer was very short. In this regard, 85.7% of the patients were discharged within 1 week after treatment. In addition, all of the patients who complained of disease-related pain before treatment experienced pain relief during and after treatment.

The toxicities observed in our study were not life-threatening. Neutropenia was the most common acute hematological toxicity. However, Grade 3 or worse neutropenia could be recovered promptly with G-CSF. Thrombocytopenia was predominantly mild to moderate (up to Grade 2). The most major acute non-hematological toxicity was vomiting. The patients suffering from Grade 3 or worse vomiting could be controlled by antiemetics. No patients required the parental support. Although the frequencies of neutropenia and vomiting were relatively high compared with other trials, all the side effects were manageable and did not require treatment interruption or dose reduction.

The local efficacy of the chosen treatment has urged us to perform further studies using HART combined with chemotherapy for LAPC patients. Based on the results of a prospective randomized trial, gemcitabine bas become the standard first-line agent in patients with advanced pancreatic cancer (26). In radiobiological models, gemcitabine has also been observed to be a potent radiosensitizer (27). However, gemcitabine could not be used for pancreatic cancer patients when we started this study. At present, gemcitabine should be used for a chemotherapieutic reagent against advanced pancreatic cancer, and current study will be helpful to design clinical trials using combined HART with gemcitabine for patients with LAPC.

Acknowledgments

This study was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare, Japan and Scientific Research from the Japan Society for the Promotion of Science.

References

1. Michaud DS. Epidemiology of pancreatic cancer. *Minerva Chir* 2004;59:99–111.
2. Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet* 2004;363:1049–57.
3. Willett CG, Czito BG, Bendell JC, Ryan DP. Locally advanced pancreatic cancer. *J Clin Oncol* 2005;23:4538–44.
4. The Gastrointestinal Tumor Study Group. Therapy of locally unresectable pancreatic carcinoma: a randomized comparison of high dose (6000 rads) radiation alone, moderate dose radiation (4000 rads + 5-fluorouracil), and high dose radiation + 5-fluorouracil. *Cancer* 1981;48:1705–10.
5. The Gastrointestinal Tumor Study Group. Treatment of locally unresectable carcinoma of the pancreas: comparison of combined-modality therapy (chemotherapy plus radiotherapy) to chemotherapy alone. *J Natl Cancer Inst* 1988;80:751–5.
6. Saunder MI. Programming of radiotherapy in the treatment of non-small-cell lung cancer—a way to advance care. *Lancet Oncol* 2001;2:401–8.
7. Horiot JC, Bontemps P, van den Bogaert W, Le Fur R, van den Weijngaert D, Bolla M, et al. Accelerated fractionation (AF) compared to conventional fraction (CF) improves loco-regional control in the radiotherapy of advanced head and neck cancers: results of the EORTC 22851 randomized trial. *Radiother Oncol* 1997;44:111–21.
8. Saunders MI, Dische S, Barrett A, Parmar M, Harvey A, Gibson D. Continuous hyperfractionated accelerated radiotherapy (CHART) versus conventional radiotherapy in non-small-cell lung cancer: a randomised multicentre trial. *Lancet* 1997;350:161–5.
9. Schuster-Uitterhoeve AL, Gonzalez Gonzalez D, Blank LE. Radiotherapy with multiple fractions per day in pancreatic and bile duct cancer. *Radiother Oncol* 1986;7:205–13.
10. Seydel HG, Stablein DM, Leichman LP, Kinzie JJ, Thomas PR. Hyperfractionated radiation and chemotherapy for unresectable localized adenocarcinoma of the pancreas. The Gastrointestinal Tumor Study Group experience. *Cancer* 1990;65:1478–82.
11. Prott FJ, Schonekaes K, Preusser P, Ostkamp K, Wagner W, Micke O, et al. Combined modality treatment with accelerated radiotherapy and chemotherapy in patients with locally advanced inoperable carcinoma of the pancreas: results of a feasibility study. *Br J Cancer* 1997;75:597–601.

12. Luderhoff EC, Gonzalez Gonzalez D, Bakker P. Pilot study in locally advanced unresectable pancreas carcinoma using a combination of accelerated radiotherapy and continuous infusion of 5-fluorouracil. *Radiother Oncol* 1996;40:241–3.
13. Ashamalla H, Zaki B, Mokhtar B, Colella F, Selim H, Krishnamurthy M, et al. Hyperfractionated radiotherapy and paclitaxel for locally advanced/unresectable pancreatic cancer. *Int J Radiat Oncol Biol Phys* 2003;55:679–87.
14. Ueno H, Okusaka T, Ikeda M, Tokuuye K. Phase I study of hyperfractionated radiation therapy with protracted 5-fluorouracil infusion in patients with locally advanced pancreatic cancer. *Oncology* 2004;67:215–21.
15. Shirasaka T, Shimamoto Y, Ohshimo H, Saito H, Fukushima M. Metabolic basis of the synergistic antitumor activities of 5-fluorouracil and cisplatin in rodent tumor models in vivo. *Cancer Chemother Pharmacol* 1993;32:167–72.
16. Skov K, MacPhail S. Interaction of platinum drugs with clinically relevant x-ray doses in mammalian cells: a comparison of cisplatin, carboplatin, iproplatin, and tetraplatin. *Int J Radiat Oncol Biol Phys* 1991;20:221–5.
17. Sun JR, Brown JM. Lack of differential radiosensitization of hypoxic cells in a mouse tumor at low radiation doses per fraction by cisplatin. *Radiat Res* 1993;133:252–6.
18. Schaake-Koning C, van den Bogaert W, Dalesio O, Festen J, Hoogenhout J, van Houtte P, et al. Effects of concomitant cisplatin and radiotherapy on inoperable non-small-cell lung cancer. *N Engl J Med* 1992;326:524–30.
19. Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982;5:649–55.
20. US Food and Drug Administration, Center for Drug Evalution and Research. Oncology Tools, Toxicity Criteria. Available from: http://www.fda.gov/cder/cancer/toxicityframe.htm.
21. Simon R. Optimal two-stage designs for phase II clinical trials. *Control Clin Trials* 1989;10:1–10.
22. Sobin LH, Wittekind CH, editors. International Union Against Cancer (UICC). TNM Classification of Malignant Tumors. 5th edn. New York: John Wiley & Sons, Inc. 1997.
23. Lokich JJ, Ahlgren JD, Gullo JJ, Philips JA, Fryer JG. A prospective randomized comparison of continuous infusion fluorouracil with a conventional bolus schedule in metastatic colorectal carcinoma: a Mid-Atlantic Oncology Program Study. *J Clin Oncol* 1989;7:425–32.
24. Nakajima Y, Miyamoto T, Tanabe M, Watanabe I, Terasima T. Enhancement of mammalian cell killing by 5-fluorouracil in combination with X-rays. *Cancer Res* 1979;39:3763–7.
25. Smalley SR, Kimler BF, Evans RG. 5-Fluorouracil modulation of radiosensitivity in cultured human carcinoma cells. *Int J Radiat Oncol Biol Phys* 1991;20:207–11.
26. Burris HA, Moore MJ, Anderson J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997;15:2403–13.
27. Lawrence TS, Eisbruch A, Shewach DS. Gemcitabine-mediated radiosensitization. *Semin Oncol* 1997;24:S7:24–8.

Schedule-dependent Therapeutic Effects of Gemcitabine Combined with Uracil-Tegafur in a Human Pancreatic Cancer Xenograft Model

Masanori Tsujie, MD, PhD, Shoji Nakamori, MD, PhD,*† Shin Nakahira, MD,* Setsuo Takeda, PhD,* Yuji Takahashi, MD,* Nobuyasu Hayashi, MD, PhD,* Jiro Okami, MD, PhD,* Hiroaki Nagano, MD, PhD,* Keizo Dono, MD, PhD,* Koji Umeshita, MD, PhD,* Masato Sakon, MD, PhD,* and Morito Monden MD, PhD**

Objectives: Gemcitabine is taken up by cells mainly via human equilibrative nucleoside transporter 1 (hENT1). Pretreatment of cancer cell lines with 5-fluorouracil (5-FU) leads to an increase in the expression of hENT1 and augments the effect of single-agent gemcitabine treatment in vitro. The purpose of the present study was to evaluate the relationship between the schedules of gemcitabine/uracil-tegafur (UFT) combination therapy and their effects in pancreatic cancer in vivo.

Methods: The expression level of hENT1 mRNA was examined using 6 types of human pancreatic cancer cell lines treated with 5-FU and MiaPaCa-2 xenograft tumors in BALB/c nu/nu mice treated with UFT. A [^{3}H] gemcitabine cellular uptake assay was performed using MiaPaCa-2 cells treated with 5-FU. We compared the effects of 6 different schedules of treatment using UFT and/or gemcitabine on MiaPaCa-2 xenograft tumors.

Results: MiaPaCa-2 cell line was one of the lines that showed the highest rate of 5-FU-induced increase in the hENT1 mRNA level. Gemcitabine uptake was significantly increased when cells were treated with 5-FU. Treatment with UFT significantly increased the hENT1 mRNA expression in MiaPaCa-2 tumors. A significant growth inhibition of MiaPaCa-2 tumors was observed in the mice treated with UFT followed by gemcitabine as compared with either untreated mice or UFT alone-treated mice.

Conclusions: Our results suggest that the schedule in which the gemcitabine is administered after UFT may be the optimal combination for gemcitabine/UFT treatment in pancreatic cancer.

Key Words: uracil-tegafur, 5-fluorouracil, gemcitabine, human equilibrative nucleoside transporter, combination chemotherapy, pancreatic cancer

(*Pancreas* 2006;33:142–147)

Received for publication July 15, 2005; accepted March 9, 2006.

From the *Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University, E2, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; †Department of Surgery, Cancer Center, Department of Molecular Medicine, Osaka National Hospital, National Hospital Organization, 2-1-14 Hoenzaka, Chuo-ku, Osaka 540-0006, Japan.

This study was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare, Japan and Scientific Research from the Japan Society for the Promotion of Science.

Reprints: Shoji Nakamori, MD, PhD, Chief Surgeon of Department of Surgery, Cancer Center, Head of Department of Molecular Medicine, Osaka National Hospital, National Hospital Organization, 2-1-14 Hoenzaka, Chuo-ku, Osaka 540-0006, Japan (e-mail: nakamori@onh.go.jp).

Pancreatic cancer remains one of the most malignant cancers. Surgery is the only curative treatment currently available. However, at the time of diagnosis, more than 80% of patients have advanced regional disease or distant metastasis.[1] Therefore, curative resection cannot be considered for most pancreatic cancers. Chemotherapy, radiation, or a combination of these therapies most commonly play a palliative role in pancreatic cancer care and have not had a significant impact on survival rates in recent decades.[1] In randomized trials, gemcitabine was the first and only chemotherapeutic agent that has been shown to have any meaningful impact on either survival or disease-related symptoms in pancreatic cancer as compared with 5-fluorouracil (5-FU).[2] Based on the results of this trial, the systemic administration of gemcitabine is currently considered as the standard first-line treatment for patients with advanced disease. However, the median survival times, even of patients treated with gemcitabine, remain unsatisfactory. Therefore, several recent efforts have focused on evaluating chemotherapy regimens in which gemcitabine is combined with other cytotoxic drugs.[1,3,4]

The relatively mild toxicity profile of gemcitabine has allowed for the development of gemcitabine-based combination chemotherapy regimens.[3,4] Several regimens have combined gemcitabine with 5-FU.[3,4] However, no randomized Phase III trial has yet established the survival benefits of the combination of gemcitabine and 5-FU as compared with gemcitabine alone.[3,4] Moreover, few studies have focused on optimizing the efficacy of gemcitabine through modification of the schedule of these 2 reagents.

Gemcitabine is a cell cycle–dependent deoxycytidine analog of the antimetabolic class. First, it must be transported into the cell and then be phosphorylated to its active triphosphate form. Incorporation of gemcitabine triphosphate into DNA is most likely the major mechanism through which gemcitabine exerts its cytotoxic actions.[5]

Cells can synthesize nucleotides either through a de novo synthesis pathway or through a salvage pathway. As one of the thymidylate synthase (TS) inhibitors, 5-FU is known to act as a de novo synthesis inhibitor.[6] In the salvage pathway, nucleosides and nucleobases must first be transported across the cell membrane by nucleoside transporter

proteins. In addition to nucleosides, nucleoside analogs are also taken up into the cell via these specific transporters.[7] Gemcitabine is a substrate for 5 of the nucleoside transporters found in humans.[8] These are human equilibrative nucleoside transporter 1 (hENT1), hENT2, human concentrative nucleoside transporter 1 (hCNT1), hCNT2, and hCNT3. The most active gemcitabine uptake is via hENT1.[8] It has been reported that 5-FU leads to an increase in cell surface hENT1.[9,10] An increase in hENT1 can potentially augment the effect of gemcitabine because this reagent enters the cell via hENT1. In fact, it has also been reported that pretreatment of pancreatic cancer cell lines in vitro with 5-FU augmented the effects of single-agent gemcitabine treatment, whereas concurrent treatment or gemcitabine before 5-FU did not.[11] These results suggest that the effect of gemcitabine/5-FU combination therapy could be dependent on the selected treatment schedule. However, these results have not been verified by in vivo studies or in the clinical setting.

Although more convenient, oral administration of 5-FU is not feasible because of the high activity of dihydropyrimidine dehydrogenase (DPD) in the gut wall, which causes rapid metabolism of the drug.[12] To bypass this problem, uracil-tegafur (UFT), an oral fluoropyrimidine derivative, was developed as a combination of tegafur (FT, a prodrug of 5-FU) and uracil (a DPD inhibitor). Uracil-tegafur can be given orally, and the administration of this reagent simulates the effect of a continuous or protracted infusion of 5-FU.[13]

The purpose of the present study was to evaluate the relationship between the schedules of gemcitabine/UFT combination therapy and their effects in pancreatic cancer. We hypothesized that pretreatment with UFT would increase hENT1 and thus increase the cytotoxicity of gemcitabine, which enters the cell via hENT1, in pancreatic cancer.

MATERIALS AND METHODS

Cell Cultures

Six types of human pancreatic cancer cell lines were used in the present study. The AsPC1, MiaPaCa-2, and PSN1 cell lines were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). The BxPC3 cell line was obtained from the American Type Culture Collection (Rockville, MD). The PCI6 cell line was a gift from Dr H. Ishikawa (Hokkaido University, Sapporo, Japan). The KMP-4 cell line was a gift from Professor Imamura (Kyoto University, Kyoto, Japan). All cell lines were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (Nikken Biomedical Laboratory, Kyoto, Japan), supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc, Rockville, MD) and 100 units/mL each of penicillin and streptomycin.

Chemicals

The 5-FU was purchased from Kyowa Hakko Kogyo, Co (Tokyo, Japan). Uracil-tegafur was kindly provided by TAIHO Pharmaceutical, Co (Tokyo, Japan). Gemcitabine was kindly provided by Eli Lilly Pharmaceuticals (Indianapolis, IN). Both 5-FU and gemcitabine were dissolved in distilled water. The 5-FU was applied to cells at a concentration of less than 0.1% of the medium volume. Uracil-tegafur was mixed with distilled water and administered to mice using oral gavage. The [^{3}H] gemcitabine was purchased from Moravek Biochemicals, Inc (Brea, CA).

Quantitative Reverse Transcription–Polymerase Chain Reaction

A quantitative gene expression assay was performed using LightCycler (Idaho Technology, Salt Lake City, UT), as described previously.[14] The polymerase chain reaction primers used for detection of hENT1 were as follows: hENT1 forward primer 5′-AATATCTTTGACTGGTTGGG-3′ and reverse primer 5′-CAGCCATGAAGAAAATGAAC-3′. The primers for porphobilinogen deaminase (PBGD) were synthesized, as described previously.[15] A housekeeping gene, PBGD was used as an internal standard. Quantification data from each sample were analyzed using the LightCycler analysis software (Roche Diagnostics, Mannheim, Germany), as recommended by the manufacturer. Relative gene expression levels are expressed as quantified gene expression divided by quantified PBGD levels.

[^{3}H] Gemcitabine Cellular Uptake Assay

A [^{3}H] gemcitabine cellular uptake assay was performed, as described previously.[16] Cells were seeded to a flat-bottomed 24-well microplate (1×10^4/well) and incubated for 24 hours. The medium was replaced by 1 mL of fresh medium with or without 5-FU, followed by an additional 48 hours of culture. The cells were then exposed to [^{3}H] gemcitabine at a concentration of 23.9 ng/mL (1.0 μCi/mL). After a 1-hour exposure, the cells were washed 3 times in 1 mL of ice cold phosphate-buffered saline. The cells were then dissolved in 0.5 mL of 0.5% Triton X-100, and 0.4 mL-aliquots were sampled for radioactivity counting. Aliquots of 20 μL were also sampled for protein determination. The uptake level of [^{3}H] gemcitabine was expressed as radioactivity levels divided by protein concentrations, as measured by the Bradford method (Bio-Rad Laboratories, Madrid, Spain).

Cell Proliferation Assay

Cell viability was determined by the 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) method.[17] Cells were seeded in 96-well culture plates in culture medium at an optimal density. After 24 hours of incubation, the medium was replaced by fresh culture medium, containing either vehicle only or the indicated dose of test chemicals. Cells were exposed to the drug for 2 days. The MTT assay was performed according to the instructions provided by the manufacturer. Absorbance was measured at 560 nm using a microtiter plate reader (Wako, Osaka, Japan). In this method, the results only correlate with viable cell numbers. The IC_{30} and IC_{50} values were estimated for each chemical in all cell lines by plotting the rate of growth inhibition versus drug concentration.

Animal Experiments

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Medicine, Osaka University. Normal female

BALB/c nu/nu mice were obtained from Japan Clea (Tokyo), maintained in a protected environment, and provided with autoclaved food and water ad libitum. All handling of mice took place under a hood. Four-week-old BALB/c mice were used in this experiment. MiaPaCa-2 cells (5×10^6 cells, resuspended in 100 μL of phosphate-buffered saline) were injected subcutaneously into the left flanks of the mice. Tumors were distinctively visible one week after inoculation.

First, the BALB/c mice bearing distinct MiaPaCa-2 tumors were divided into 2 groups (each group, n = 6) according to tumor volume (day 0); one group was untreated (group N), whereas the other group was treated with UFT on days 1 to 5 (group T). Uracil-tegafur (24 mg/kg) was administered by oral gavage. The dose of UFT was decided based on the previous reports.[18] On day 6, quantitative gene expression of hENT1 mRNA in MiaPaCa-2 tumors was analyzed using LightCycler.

Next, mice bearing distinct tumors were divided into 6 groups (A–F, 6 mice in each group) according to tumor volume (day 0) (Fig. 1). Group A was untreated (control). Group B was treated with gemcitabine alone (administered on days 3 and 10). Group C was treated with UFT alone. Group D was treated with gemcitabine followed by UFT (gemcitabine was administered on days 0 and 7). Group E was concurrently treated with gemcitabine and UFT (gemcitabine was administered on days 3 and 10). Group F was treated with UFT followed by gemcitabine (gemcitabine was administered on days 6 and 13). Uracil-tegafur was administered on days 1 to 5 and days 8 to 12 in all UFT-treated groups (C, D, E, and F). Gemcitabine (240 mg/kg) was administered by intraperitoneal injection, and UFT (24 mg/kg) was administered by oral gavage. The dose of UFT or gemcitabine was decided based on the previous reports.[18,19] Tumors were measured with a caliper every 3 to 4 days, and the volume was calculated as length × width^2 × 0.5. Mice were weighed every 3 to 4 days using an electronic balance. Statistical analysis of the data for the comparison of different groups was carried out using tumor volumes on day 25.

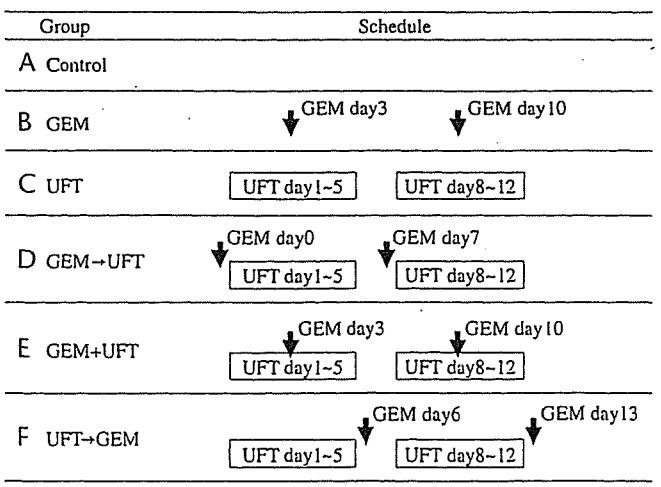


FIGURE 1. Treatment schedule. MiaPaCa-2 cells (5×10^6 cells/100 μL phosphate-buffered saline) were injected subcutaneously into the left flanks of BALB/c mice. The mice were divided into 6 groups of 6 mice according to tumor volume. Gemcitabine (240 mg/kg) was administered by intraperitoneal injection, and UFT (24 mg/kg) was administered by oral gavage. GEM indicates gemcitabine.

TABLE 1. IC_{50} Values for 5-FU in 6 Types of Human Pancreatic Cancer Cell Lines

Cell Line	IC_{50} (μgmL)
AsPC1	33.2 ± 2.4
BxPC3	1.7 ± 0.2
MiaPaCa-2	2.3 ± 0.2
PSN1	1.7 ± 0.3
PC16	6.3 ± 0.3
KMP-4	2.0 ± 0.3

Data are expressed as mean ± SE.

Statistical Analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student *t* test if the distribution of samples was normal or the Mann-Whitney *U* test if the sample distribution was asymmetrical. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

IC_{50} of 5-FU in Human Pancreatic Cancer Cell Lines

First, we examined the IC_{50} values of 5-FU in 6 human pancreatic cancer cell lines (Table 1). AsPC1 cells showed the highest IC_{50} value (33.2 ± 2.4 μg/mL). The IC_{50} values in BxPC3 and PSN1 were the lowest (1.7 ± 0.2 and 1.7 ± 0.3 μg/mL, respectively).

hENT1 mRNA Expression in Pancreatic Cancer Cells Treated With 5-FU

We examined the effects of 5-FU on the expression of hENT1 mRNA in 6 types of human pancreatic cancer cell

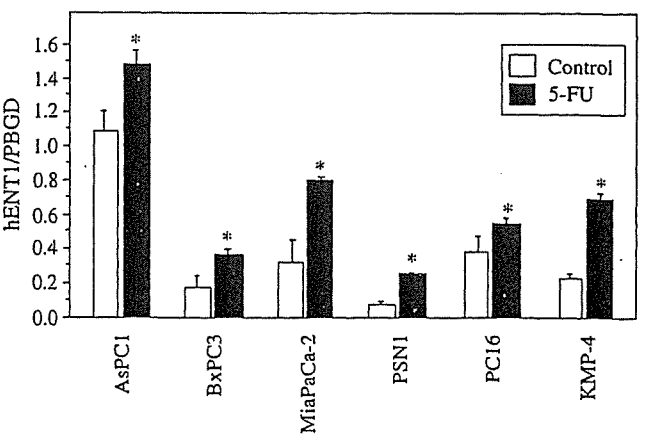


FIGURE 2. Quantification of hENT1 mRNA expression in pancreatic cancer cells treated with 5-FU. In the treatment group, cells were treated with 5-FU at a concentration of IC_{50} for 2 days. Data are expressed as mean ± SD for the 3 experiments. *$P < 0.05$ as compared with untreated control.

lines using a quantitative reverse transcriptase–polymerase chain reaction assay. Each cell line was treated with 5-FU at IC_{50} concentration. As shown in Figure 2, treatment with 5-FU resulted in a significant increase in hENT1 mRNA expression as compared with untreated cells in all lines tested. Of the 6 lines, MiaPaCa-2, PSN1, and KMP-4 cells showed relatively high rates of 5-FU–induced increase in hENT1 mRNA level. Although AsPC1 cells showed the highest expression levels, the rate of 5-FU–induced increase was lower than above 3 lines.

Uptake of Gemcitabine in Pancreatic Cancer Cells

To evaluate the uptake level of gemcitabine in pancreatic cancer cells treated with 5-FU, a [^{3}H] gemcitabine cellular uptake assay was performed (Fig. 3). The 5-FU treated cells showed increased [^{3}H] gemcitabine uptake in a dose-dependent manner. The uptake levels of the cells treated with 5-FU at IC_{30} or IC_{50} concentrations were significantly higher than those of the untreated cells.

hENT1 mRNA Expression in Pancreatic Cancer Xenograft Tumors

For in vivo experiments, we selected 3 cell lines (MiaPaCa-2, PSN1, and KMP-4 cell lines) which showed the higher rate of 5-FU–induced increase in hENT1 mRNA. However, we failed to establish subcutaneous tumors using PSN1 and KMP-4 cell lines in BALB/c nu/nu mice. Therefore, we used MiaPaCa-2 cells for the following in vivo experiments.

We examined the effect of UFT on the hENT1 mRNA expression of xenograft tumors in BALB/c mice. The quantified hENT1 expression level of group T (treated with UFT for 5 days) was significantly higher than that of group N (untreated control; Fig. 4), whereas its expression levels after gemcitabine treatment of concurrent treatment were not examined.

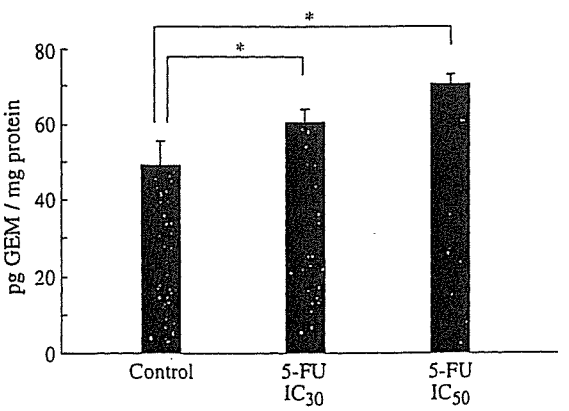


FIGURE 3. Uptake of gemcitabine in MiaPaCa-2 cells treated with 5-FU. In the 5-FU treatment group, cells were treated with different concentrations (IC_{30} and IC_{50}) of 5-FU for 2 days and then treated with [^{3}H] gemcitabine for an hour. Data are expressed as mean ± SD for the 3 experiments. *$P < 0.05$ as compared with untreated control. Abbreviations are explained in the first footnote to Figure 1.

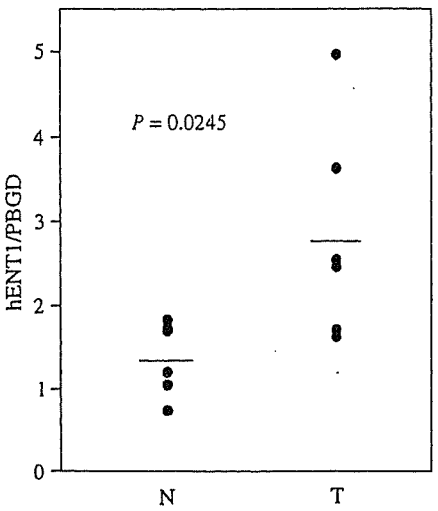


FIGURE 4. Quantification of hENT1 mRNA expression in MiaPaCa-2 xenograft tumors treated with or without UFT. BALB/c mice bearing distinct tumors were divided into 2 groups; one group was untreated (N), whereas the other group was treated with UFT for 5 days (T). Uracil-tegafur (24 mg/kg) was administered by oral gavage. The horizontal line represents the mean value of each group.

Optimal Schedule of UFT/Gemcitabine Combination Therapy In Vivo

We compared the different schedules of treatment of UFT and/or gemcitabine against MiaPaCa-2 xenograft tumors in BALB/c mice to clarify the optimal schedule for these 2 reagents. Six different groups were subjected to the therapeutic experiments (see the "Materials and Methods" section for details). Statistical analysis for the comparison of different groups was performed using tumor volumes on day 25. The mean tumor volumes on day 25 were 2.1 ± 1.1, 1.2 ± 0.6, 1.6 ± 0.7, 1.2 ± 0.5, 1.0 ± 0.7, and 0.7 ± 0.5 cm^3 in groups A, B, C, D, E, and F, respectively (Fig. 4). Of the 6 groups, the average tumor size of group F (UFT followed by gemcitabine) had the tendency to be the smallest, and statistically significant growth inhibition was observed in group F as compared with group A (control, $P = 0.0171$) and group C (UFT alone, $P = 0.0340$) (Fig. 5). Moreover, the synergistic effect was observed only in group F among the 3 combination groups. Although the mice which were given UFT first or simultaneously with gemcitabine showed stronger weight loss as compared with other groups during and after the treatment, no mice died from the side effects of the drugs or any other reason up to the end of the study (day 25) (Fig. 6).

DISCUSSION

The combination of anticancer agents has become a highly important modality for the treatment of cancer. Furthermore, combination chemotherapy is influenced by the use of optimal scheduling.[20] Gemcitabine is considered to be attractive for combination chemotherapy because of its mild toxicity profile at an active dose.[3,4] However, in one study, the combination of 5-FU with gemcitabine did not improve the

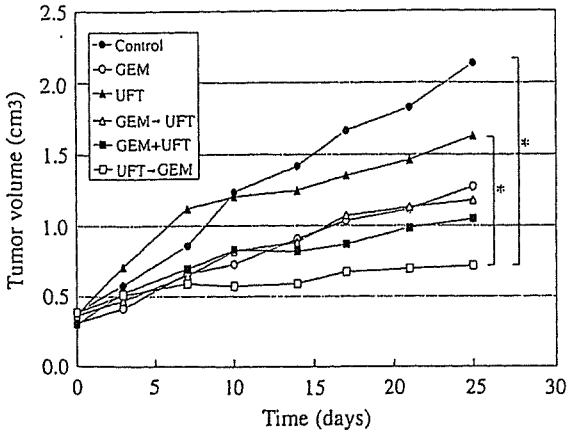


FIGURE 5. Antitumor effect of the combination of gemcitabine with UFT in vivo. Six different groups of the therapeutic experiments were carried out (Fig. 1). Statistical analysis for the comparison of different groups was performed using tumor volumes on day 25. Data are expressed as means of calculated tumor sizes (each group, n = 6). *$P < 0.05$. Abbreviations are explained in the first footnote to Figure 1.

median survival of patients with advanced pancreatic carcinoma as compared with single-agent gemcitabine.[21] Moreover, another report showed a significant increase in toxicity in 5-FU and folinic acid plus gemcitabine group as compared with the 5-FU and folinic acid alone group.[22] Therefore, we evaluate the effect of gemcitabine/UFT combination therapy on human pancreatic cancer, especially in the schedule of 2 drugs. Our study shows that the sequence can be an important factor in the antitumor activity of the combination of gemcitabine and UFT and that hENT1 might play an important role in this effect.

We first examined the effects of 5-FU on expression of hENT1 mRNA in pancreatic cancer cell lines. Known as a TS inhibitor, 5-FU blocks the formation of 2-deoxythymidine-5-monophosphate and depletes intracellular nucleoside pools so that proliferating cells then depend on the salvage of preformed nucleosides from extracellular fluid. As confirmed by flow cytometric analysis, 5-FU also up-regulates the amount of cell surface hENT1.[11] In this study, we demonstrated that treatment with 5-FU resulted in increased hENT1 expression at mRNA level using a quantitative reverse transcriptase–polymerase chain reaction method.

It has been reported that the sensitivity to nucleoside analogs correlates with the expression of hENT1 and that the hENT1-deficient cells are highly resistant to nucleoside analogs.[8] Our recent in vitro experiments using 5-FU and gemcitabine also suggested that an interaction based on the up-regulation of hENT1 might work as one of the mechanisms for making gemcitabine more effective (data not shown). However, we did not detect any relationship between sensitivity to gemcitabine and the expression level of hENT1 in pancreatic cancer cell lines (data not shown). Gemcitabine is transported also via other nucleoside transporters (e.g., hCNT1). Inside the cell, this drug is converted by deoxycytidine kinase to its triphosphate, which is incorporated into DNA and terminates DNA strand elongation, resulting in apoptosis. Therefore, although hENT1 plays an important role in gemcitabine uptake, other factors, such as other nucleoside transporters, deoxycytidine kinase activation, and apoptosis-regulating genes may have possibilities to be more critical in determining gemcitabine sensitivity in pancreatic cancer cells.[23–26]

For the in vivo experiment, we chose UFT instead of 5-FU because UFT has several advantages over 5-FU in the treatment of tumors. First, UFT can be given orally, which is more convenient in a clinical setting. Second, administration of UFT results in a higher 5-FU concentration in the tumor tissues than comparable doses of intravenous 5-FU.[27] Third, the combination of FT and DPD inhibitor for the treatment of MiaPaCa-2 cells was reported to show a greater antitumor effect than treatment with FT alone in an in vivo study.[28] Lastly, it was also reported that the 5-FU concentration in the tumor tissues in patients treated with UFT is greater than that in the normal tissue and that UFT is considered to be well tolerated in an in vivo and clinical setting.[29]

The sequence-dependent effects of the combination of TS inhibitors and gemcitabine on human pancreatic cancer cells were reported to be seen with maximum effect when the TS inhibitors preceded gemcitabine in vitro.[11] Moreover, this effect was not associated with basal hENT1 levels but with a significant increase in hENT1 levels caused by the TS inhibitors.[11] Therefore, for in vivo experiments, we decided to use the cell lines which showed the highest rate of 5-FU–induced increase in hENT1 mRNA. In this study, we first showed that treatment with UFT resulted in a significant increase of hENT1 mRNA expression in xenograft tumors. To clarify the optimal schedule of treatment, we investigated the effect of 6 different schedules of treatment of UFT and/or gemcitabine on xenograft model of MiaPaCa-2 tumors. We did not detect any significant differences among the 3 gemcitabine/UFT combination groups (UFT followed by gemcitabine, concurrent treatment of gemcitabine and UFT, and gemcitabine followed by UFT). However, significant growth inhibition was observed in mice treated with UFT followed by gemcitabine as compared with either untreated mice or those treated with UFT alone. Moreover, the synergistic growth inhibitory effect was observed only in UFT followed by gemcitabine group and not in other combination groups. These results suggest that the

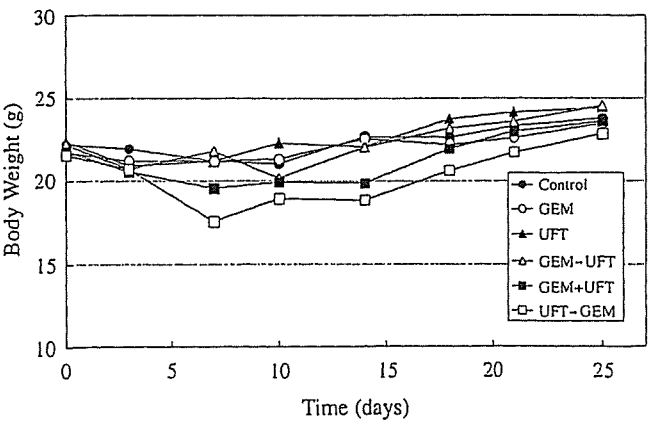


FIGURE 6. Body weight curves of the mice. The corresponding tumor growth curves are shown in Figure 5.

schedule in which the gemcitabine is administered after UFT could be the optimal combination of these 2 reagents in the treatment of pancreatic cancer. The up-regulation of hENT1 induced by UFT may play an important role in the enhanced growth inhibitory effect of gemcitabine. Moreover, our preliminary results with another oral fluoropyrimidine derivative also suggested that pretreatment of oral fluoropyrimidine derivative before gemcitabine administration provided greater inhibitory effects than the other gemcitabine/fluoropyrimidine derivative schedules in the treatment of pancreatic cancer in in vivo experiments.

Previous reports showed that treatment of gemcitabine increased hENT1 expression and reduced 5-FU sensitivity in human pancreatic cancer cell lines.[11] Therefore, the pretreatment with gemcitabine might reduce the effects of 5-FU because of the increasing supply of nucleosides and nucleobases via salvage pathway. Indeed, the expression level of basal hENT1 was reversely associated with 5-FU sensitivity in 6 types of human pancreatic cancer cell lines used in this study (Fig. 2 and Table 1). These data suggested that the lack of benefit with sequences other than UFT followed by gemcitabine in vivo might be caused by the gemcitabine-induced up-regulation of hENT1 and subsequently reduced 5-FU sensitivity.

It has been reported that mouse ENT1 mRNA was highly expressed in the heart, spleen, lung, liver, and testis and that lower levels of expression were detected in the brain and kidney.[30] Therefore, enhanced gemcitabine effects induced by UFT may also aggravate gemcitabine-induced side effects. However, in this study, neither severe side effects nor mortality was observed, although the mice treated with UFT first or simultaneously with gemcitabine showed more weight loss than mice in the other groups.

In conclusion, although further research including clinical studies is needed, our results showed that the administration of UFT followed by gemcitabine provides greater inhibitory effects than the administration of other gemcitabine/UFT schedules in the treatment of pancreatic cancer.

REFERENCES

1. Li D, Xie K, Wolff R, et al. Pancreatic cancer. *Lancet.* 2004; 363:1049–1057.
2. HA Burris 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol.* 1997;15:2403–2413.
3. Kulke MH. Advanced pancreatic cancer: is there a role for combination therapy? *Expert Rev Anticancer Ther.* 2003;3:729–739.
4. El-Rayes BF, Philip PA. Systemic therapy for advanced pancreatic cancer. *Expert Rev Anticancer Ther.* 2002;2:426–436.
5. Huang P, Chubb S, Hertel LW, et al. Action of 2′, 2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res.* 1991;51: 6110–6117.
6. Thomas DM, Zalcberg JR. 5-fluorouracil: a pharmacological paradigm in the use of cytotoxics. *Clin Exp Pharmacol Physiol.* 1998;25:887–895.
7. Baldwin SA, Mackey JR, Cass CE, et al. Nucleoside transporters: molecular biology and implications for therapeutic development. *Mol Med Today.* 1999;5:216–224.
8. Mackey JR, Mani RS, Selner M, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. *Cancer Res.* 1998;58:4349–4357.
9. Pressacco J, Wiley JS, Jamieson GP, et al. Modulation of the equilibrative nucleoside transporter by inhibitors of DNA synthesis. *Br J Cancer.* 1995;72:939–942.
10. Pressacco J, Mitrovski B, Erlichman C, et al. Effects of thymidylate synthase inhibition on thymidine kinase activity and nucleoside transporter expression. *Cancer Res.* 1995;55:1505–1508.
11. Rauchwerger DR, Firby PS, Hedley DW, et al. Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. *Cancer Res.* 2000;60:6075–6079.
12. Diasio RB. Clinical implications of dihydropyrimidine dehydrogenase inhibition. *Oncology (Huntingt).* 1999;13:17–21.
13. Hoff PM, Royce M, Medgyesy D, et al. Oral fluoropyrimidines. *Semin Oncol.* 1999;26:640–646.
14. Miyamoto A, Nagano H, Sakon M, et al. Clinical application of quantitative analysis for detection of hematogenous spread of hepatocellular carcinoma by real-time PCR. *Int J Oncol.* 2000;18: 527–532.
15. Finke J, Fritzen R, Temes P, et al. An improved strategy and a useful housekeeping gene for RNA analysis from formalin-fixed, paraffin-embedded tissues by PCR. *Biotechniques.* 1993;14:448–453.
16. Garcia-Manteiga J, Molina-Arcas M, Casado FJ, et al. Nucleoside transporter profiles in human pancreatic cancer cells: role of hCNT1 in 2′, 2′-difluorodeoxycytidine-induced cytotoxicity. *Clin Cancer Res.* 2003;9:5000–5008.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1993;65:55–63.
18. Fukushima M, Satake H, Uchida J, et al. Preclinical antitumor efficacy of S-1: a new oral formulation of 5-fluorouracil on human tumor xenografts. *Int J Oncol.* 1998;13:693–698.
19. Braakhuis BJ, Ruiz van Haperen VW, Boven E, et al. Schedule-dependent antitumor effect of gemcitabine in in vivo model system. *Semin Oncol.* 1995;22(11 suppl):42–46.
20. Goldin A. Combined chemotherapy. *Oncology.* 1980;37(suppl 1):3–8.
21. Berlin JD, Catalano P, Thomas JP, et al. Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. *J Clin Oncol.* 2002;20:3270–3275.
22. Correale P, Cerretani D, Marsili S, et al. Gemcitabine increases systemic 5-fluorouracil exposure in advanced cancer patients. *Eur J Cancer.* 2003;39:1547–1551.
23. Garcia-Manteiga J, Molina-Arcas M, Casado FJ, et al. Nucleoside transporter profiles in human pancreatic cancer cells: role of hCNT1 in 2′, 2′-difluorodeoxycytidine–induced cytotoxicity. *Clin Cancer Res.* 2003;9:5000–5008.
24. Ruiz van Haperen VW, Veerman G, Eriksson S, et al. Development and molecular characterization of a 2′, 2′-difluorodeoxycytidine–resistant variant of the human ovarian carcinoma cell line A2780. *Cancer Res.* 1994;54:4138–4143.
25. Xu Z, Friess H, Solioz M, et al. Bcl-x (L) antisense oligonucleotides induce apoptosis and increase sensitivity of pancreatic cancer cells to gemcitabine. *Int J Cancer.* 2001;94:268–274.
26. Shi X, Liu S, Kleeff J, et al. Acquired resistance of pancreatic cancer cells towards 5-fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. *Oncology.* 2002;62:354–362.
27. Takiuchi H, Ajani JA. Uracil-tegafur in gastric carcinoma: a comprehensive review. *J Clin Oncol.* 1998;16:2877–2885.
28. Takechi T, Fujioka A, Matsushima E, et al. Enhancement of the antitumour activity of 5-fluorouracil (5-FU) by inhibiting dihydropyrimidine dehydrogenase activity (DPD) using 5-chloro-2, 4-dihydroxypyridine (CDHP) in human tumour cells. *Eur J Cancer.* 2002;38:1271–1277.
29. Taguchi T. Clinical application of biochemical modulation in cancer chemotherapy: biochemical modulation for 5-FU. *Oncology.* 1997;54 (1 suppl):12–18.
30. Choi DS, Handa M, Young H, et al. Genomic organization and expression of the mouse equilibrative, nitrobenzylthioinosine-sensitive nucleoside transporter.1 (ENT1) gene. *Biochem Biophys Res Commun.* 2000;277:200–208.

Clinical Roles of Increased Populations of Foxp3$^+$CD4$^+$ T Cells in Peripheral Blood from Advanced Pancreatic Cancer Patients

Tetsuya Ikemoto, MD, PhD,† Takeshi Yamaguchi, MD,† Yuji Morine, MD, PhD,† Satoru Imura, MD, PhD,† Yuji Soejima, MD, PhD,† Masahiko Fujii, MD, PhD,† Yoichi Maekawa, MD, PhD,* Koji Yasutomo, MD, PhD,* and Mitsuo Shimada, MD, PhD†*

Objectives: Further metastasis should be avoided in pancreatic cancer (PC) patients for effective surgical treatment. Regulatory T cells (Foxp3$^+$ CD4$^+$ T cells including CD4$^+$CD25$^+$ T cells and CD4$^+$CD25$^-$ T cells) play important roles in tumor immunity. This study aimed to investigate whether regulatory T cells participate in metastasis.

Methods: Peripheral blood was withdrawn from PC patients, as well as healthy volunteer donors as controls. The peripheral blood mononuclear cells (PBMCs) were subjected to FACScan analysis after labeling with anti-CD4, anti-CD25, and anti-Foxp3 antibodies. Tumor markers, including DUPAN2 and CA19-9, surface markers, such as the CD4/CD8 ratio, and the CD57$^+$ cell population were assessed. Clinical stages were classified according to the TNM classification.

Results: The Foxp3$^+$CD4$^+$ T-cell population among the PBMCs was significantly increased in PC patients (8.10% ± 4.65%) compared with healthy donors (2.47 ± 0.78%) ($P < 0.001$). No significant relationships existed for the tumor markers, CD4/CD8 ratio, and CD57$^+$ cells. However, a significant correlation was found between Foxp3$^+$CD4$^+$ T cells among the PBMCs and the TNM stage ($P < 0.05$).

Conclusions: Foxp3$^+$CD4$^+$ T cells are good markers for metastasis detection in PC patients and more accurate than other conventional tumor markers, especially at advanced stages of the disease.

Key Words: pancreatic cancer, Foxp3, regulatory T cells, tumor immunity

(*Pancreas* 2006;33:386–390)

Pancreatic cancer (PC) is the fifth most common malignancy in Japan and the fourth in the United States and is known to contribute to the increasing numbers of cancer deaths. Despite advances in therapies, it remains difficult to detect PC, diagnose the disease, and radically cure it.

Previous studies on PC have shown that only complete curative surgical resection can dramatically prolong the patient prognosis, despite adjuvant therapies such as chemotherapy, radiation, and so on.[1,2] Therefore, it is important to diagnose PC at an early stage to allow for curative resection. The present study aimed to investigate invasion by PC cells that escape the normal immune system, to accomplish curative resection. Initially, we assessed patients with advanced PC to identify differences between their host immune systems and those of noncancer patients. If such differences exist, the various stages of PC may show differences in the host immune systems, despite the histological malignancy of PC itself. To estimate the states of the host immune system, we assessed regulatory T (regT) cells. RegT cells are regulators and suppressors of T cells,[3,5] can induce a tolerant state in transplantation,[4,16] are found in various autoimmune diseases,[5] and regulate tumor immunity.[6,15] Naturally occurring CD4$^+$ regT cells are generally identified by their expression of CD25. Moreover, the numbers of CD4$^+$CD25$^+$ T cells increase during T-cell activation.[7,8] However, it remains unclear whether CD4$^+$CD25$^+$ T cells exhibit a truly regulatory activity. Foxp3 is a forkhead/winged helix transcription factor, also known as scurfin, which was reported to be a key regulatory gene for the development of regT cells.[7] On the other hand, it has also been reported that some CD4$^+$CD25$^-$ T cells show powerful regulatory activity toward regT cell development.[9] Thus, Foxp3$^+$CD4$^+$ T cells can be referred to as "potential" regT cells. Here, we investigated whether Foxp3$^+$CD4$^+$ T cells can be used to predict the state of the immune system in patients, particularly patients with advanced PC.

MATERIALS AND METHODS

Background of Patients and Normal Donors

Peripheral blood was withdrawn from patients with pancreatic cancer (PC group, n = 16) as well as healthy donors (HD group, n = 27) as a control group. The latter group consisted of age- and sex-matched healthy individuals. Informed consent was obtained from all patients and healthy donors according to the Declaration of Helsinki.[10] Furthermore, none of the subjects had received any treatments, such as surgery, chemotherapy/radiation therapy, or immunosuppressive

Received for publication February 15, 2006; accepted July 25, 2006.

From the Departments of *Immunology and Paracytology and †Digestive and Pediatric Surgery, Institute of Health Bioscience, Graduate School of Medicine, The University of Tokushima, Kuramoto, Tokushima, Japan.

This study was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (grant nos. 17790918-00 to T.I. and 17591407 to Y.T., K.Y., T.I.) and grants from the Cancer Research Project Cooperative of TAIHO Pharmaceutical Co. Ltd. and The University of Tokushima.

Reprints: Tetsuya Ikemoto MD, PhD, Department of Digestive and Pediatric Surgery, Institute of Health Bioscience, Graduate School of Medicine, The University of Tokushima, 3-18-15 Kuramoto, Tokushima, 770-8503, Japan (e-mail: tikemoto@clin.med.tokushima-u.ac.jp).

therapy, within 30 days of the specimen collection or had any histories, symptoms and/or signs of systematic/local infections, allergies, or autoimmune diseases.

Cell Isolation

Peripheral blood samples were subjected to FACScan analysis for multicolor analysis of T cells stained according to the manufacturer's guidelines (the manufacturers were described below). Briefly, a 3-mL peripheral blood sample was taken from each subject, and the peripheral blood mononuclear cells (PBMCs) were isolated using 4 mL of Lymphoprep (AXIS-SHIELD, Oslo, Norway), centrifuged (1800 rpm for 20 minutes at room temperature), and then resuspended with 4 mL of 0.9% NaCl. After addition of anti-CD4 (Allophycocyanin (APC)-conjugated antihuman CD4; e-Bioscience, San Diego, Calif) and anti-CD25 (fluorescein isothiocyanate–conjugated antihuman CD25; e-Bioscience) antibodies to the cell pellets, the mixture was centrifuged at 2000 rpm for 5 minutes at 4°C, placed on ice for 15 minutes in the dark, and further incubated at −20°C overnight. After 2-time washes, blocking buffer was added, and the mixture was incubated in the dark at room temperature for 15 minutes. Next, an anti-Foxp3 antibody (Phycoerythrin (PE)-conjugated monoclonal antihuman Foxp3; e-Bioscience) was added to the cell pellet in

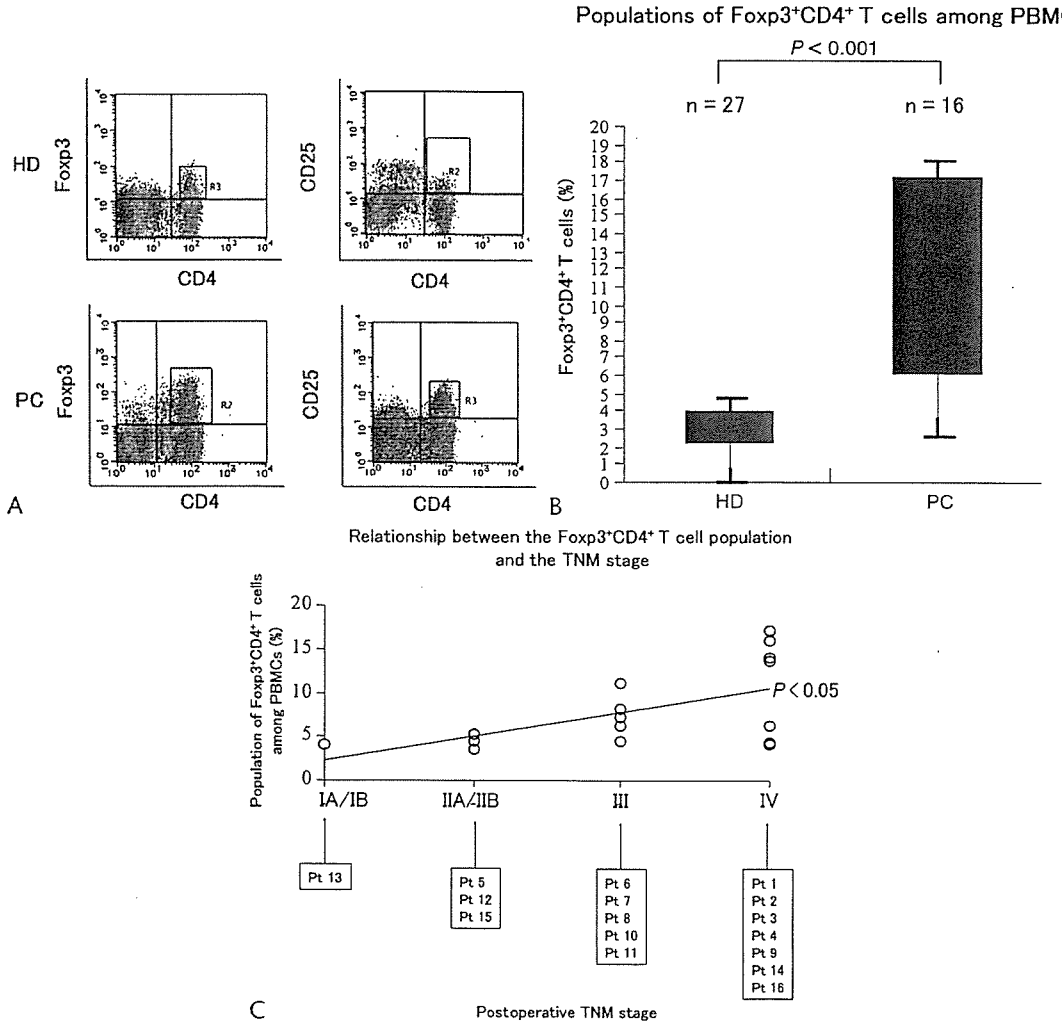


FIGURE 1. Foxp3⁺CD4⁺ lymphocytes are elevated in peripheral blood from patients with PC. A, Representative flow cytometry results of PBMCs from HD and PC patients. Isolated PBMCs labeled with APC-conjugated antihuman CD4 and PE-conjugated antihuman Foxp3 antibodies are shown. B, Cumulative results from 27 HD and 16 advanced PC patients. There are significant differences between the populations of Foxp3⁺CD4⁺ T cells among the PBMCs from HD and PC ($P < 0.001$, t test). The populations (average ± SD) of the Foxp3⁺CD4⁺ T cells among the PBMCs are shown. C, Depiction of the data in B in a scatter graph format. The postoperative clinical (pathological) stages are also shown. The trend seems to be that more localized PCs are associated with lower percentages of Foxp3⁺CD4⁺ T cells. There is a significant correlation between the population of Foxp3⁺CD4⁺ T cells among the PBMCs and the TNM stage ($P < 0.05$, Pearson test). The patient numbers correspond to those in Table 1. HD indicates healthy donors; PC, pancreatic cancer patients.

TABLE 1. Clinical Characteristics of the Study Patients

Patient	Age (y)	Sex	Stage (TNM)	Operating Findings/ Inoperable Factors	Operation	CA19-9 (U/mL)	DUPAN2 (U/mL)	CD4/CD8 of PBMCs	CD57 in PBMCs (%)
1	62	M	T4 N1 M1; stage IV	M1 (retroperitoneum)	Test laparotomy	919	420	1.02	20.6
2	68	F	T3 N1 M1; stage IV	Peritonitis carcinomatosa (cytology positive)	Not done	17,558	1400	1.07	12.4
3	62	M	T3 N1 M1; stage IV	Multiple liver metastases	Test laparotomy	<5	880	1.24	18.4
4	60	F	T3 N1 M1; stage IV	Multiple liver/lung metastasis	Not done	394	37	2.67	17.2
5	57	M	T2 N1 M0; stage IIB	Resectable	PpPD	68	49	—	—
6	68	M	T4 N1 M0; stage III	Celiac axis, portal vein invasion	DP	12,691	960	1.64	18.7
7	76	F	T4 N1 M0; stage III	Celiac axis and SMA invasion	Intraoperative radiation	22	<25	1.93	24.4
8	63	M	T4 N1 M0; stage III	Celiac axis and SMA invasion	Intraoperative radiation	394	35	2.95	11.4
9	56	F	T2 N1 M1; stage IV	Peritonitis carcinomatosa	Test laparotomy	20	520	0.88	31.7
10	77	M	T4 N1 M0; stage III	Celiac axis and SMA invasion	Intraoperative radiation	772	130	1.08	36.7
11	77	F	T4 N1 M0; stage III	Celiac axis and SMA invasion	Intraoperative radiation	287	>1600	2.68	12.6
12	64	M	T3 N1 M0; stage IIB	Resectable	PpPD	761	310	1.58	23.7
13	53	F	T2 N0 M0; stage IB	Resectable	PD	540	140	1.80	8.7
14	73	F	T2 N1 M1; stage IV	Peritonitis carcinomatosa	Test laparotomy	46	27	1.41	26.7
15	67	M	T2 N1 M0; stage IIB	Resectable	PD	761	310	2.54	28.8
16	73	M	T2 N1 M1; stage IV	Peritonitis carcinomatosa	Test laparotomy	1392	280	1.77	30.2

The tumor stage is classified according to the TNM classification. The cutoff limits of the tumor markers are 47 U/mL for CA19-9 and 150 U/mL for DUPAN2. The normal ranges of the lymphocyte surface markers in PBMCs are 1.47 ± 0.84 (CD4/CD8 ratio) and 22.1% ± 11.3% (CD57).

Em-dash (—) indicates not estimated in patient 5; DP, distal pancreatectomy; PD, pancreaticoduodenectomy; PpPD, pylorus-preserving pancreaticoduodenectomy; SMA, superior mesenteric artery.

the dark and incubated at room temperature for 30 minutes. The cells were then washed twice with FACS buffer, centrifuged at 1800 rpm for 5 minutes at 4°C, and analyzed using a FACSCalibur (BD Biosciences, San Jose, Calif) for measurement of $Foxp3^+CD4^+$ T cells and $CD4^+CD25^+$ T cells with multicolor staining. The whole procedure was achieved within 24 hours after withdrawal of the blood samples.

Other Laboratory Data and Clinical Stages

The available laboratory data for the patients with PC included estimations of tumor markers, such as CA19-9 and DUPAN2 (none of the PC group had obstructive jaundice or any signs of active cholangitis), lymphocytes surface markers, such as the CD4/CD8 ratio, and the $CD57^+$ cell population. The clinical stages of the patients were classified according to the TNM classification.[11] Pathological examinations were performed for the resected (or biopsy) specimens.

Operation

In total, 14 of 16 patients in the PC group underwent surgery. Patients 1, 3, 9, 14, and 16 underwent a test laparotomy (including tumor biopsy). Patient 2 did not undergo any surgical procedures because she was diagnosed as having peritonitis carcinomatosa from cytology of ascites. Patient 4 was also inoperable because multiple liver and lung metastases were found by multidetector-row computed tomography. Patients 7, 8, 10, and 11 agreed to undergo intraoperative radiation therapy (2500 rad [25 Gy]) because of local advanced cancer. Patient 6 underwent distal pancreatectomy and lymph node dissections with resection of the celiac axis and portal vein, and curative resection was accomplished. Patients 5, 12, 13, and 15 underwent pancreaticoduodenectomy (patients 5 and 12 accepted pylorus-preserving pancreaticoduodenectomy), and all operations accomplished curative resection.

RESULTS

$Foxp3^+CD4^+$ T-Cell Population Is Increased in Advanced Pancreatic Cancer Patients

The population of $Foxp3^+CD4^+$ T cells among the PBMCs (Fig. 1A) was significantly increased in PC patients (8.10% ± 4.65%) compared with HD (2.47% ± 0.78%) ($P < 0.001$, t test; Fig. 1B).

No Significant Correlations Between $Foxp3^+CD4^+$ T Cells Among PBMCs and Tumor Markers

In PC patients, no significant relationships were identified between $Foxp3^+CD4^+$ T cells among the PBMCs and tumor markers (CA19-9, $P = 0.1722$; DUPAN-2, $P = 0.3662$; Table 1).

No Significant Correlations Between $Foxp3^+CD4^+$ T Cells Among PBMCs and the CD4/CD8 Ratio or $CD57^+$ Cell Population

In PC patients, no significant relationships were found between $Foxp3^+CD4^+$ T cells among the PBMCs and the CD4/CD8 ratio or $CD57^+$ cell population (Table 1).

Significant Correlation Between Foxp3$^+$CD4$^+$ T Cells Among PBMCs and TNM Stage

In PC patients, a significant correlation was found between the population of Foxp3$^+$CD4$^+$ T cells among the PBMCs and the TNM stage ($P < 0.05$, Pearson test; Fig. 1C). In particular, patients 6, 7, 8, 10, and 11 were diagnosed as stage IV (M1) preoperatively. However, all 5 of these patients were classified as stage III after their tumors were located during intraoperative diagnosis. As a result, patients 7, 8, 10, and 11 underwent intraoperative radiation (2500 rad [25 Gy]), whereas patient 6 underwent a radical operation including resection of the celiac axis and portal vein. The numbers of Foxp3$^+$CD4$^+$ T cells in these patients were increased compared with the control HD group, although the numbers were rather low among the other inoperable patients whose cancer progressed to advanced forms (existence of further metastases, M1, stage IV; Fig. 1C).

DISCUSSION

It is well known that cancer cells can escape from the normal immune system. Previous reports have revealed that cancer cells may not express the APC antigen and that cancer cells can suppress/induce T-cell activation (anergy) state.[14] Moreover, some reports have shown that regT cells induce hyporesponsiveness of cancer cells,[5,6] whereas another study indicated that cancer cells themselves induce regT cells that suppress peripheral T-cell activation.[12]

On the other hand, PC has continued to result in increasing numbers of cancer deaths, despite progress in chemotherapy/radiation therapy. It is believed that chemotherapy regimens based on gemcitabine are the only hopeful option for patients with advanced PC, although various new regimens have been considered.[17,18] However, the prognosis of such patients is still not prolonged satisfactorily. The highest cure rate occurs when the cancer is specifically localized to the pancreas, suggesting that curative surgical resection is the only way to prolong the prognosis of PC patients.[1]

Thus, we first investigated the host immune states of our cancer patients to assess whether different immune states exist, to detect stages for surgical resection using regT cells. For extreme examples, we chose patients with advanced PC (stages I, II, III, and IV, not including stage 0, according to the TNM classification[11]). Although only a small number of patients were used to estimate the differences between Foxp3$^+$CD4$^+$ T cells and the stages of PC, a significant correlation was found with a tendency that higher populations of Foxp3$^+$CD4$^+$ T cells were associated with more severe metastases. However, this may not be related to tumor volume in the patients, thereby leading to the lack of significant relationships between the population of Foxp3$^+$ CD4$^+$ T cells and tumor markers, including DUPAN-2 and CA19-9, in our PC patients.

On the contrary, patients 6, 7, 8, 10, and 11 were diagnosed as having stage IV (M1) preoperatively because of suspicion of having direct invasion to the stomach with the existence of peritonitis carcinomatosa from multidetector-row computed tomography (patient 6, T4 N1 M1) and suspected tumor invasion into the celiac axis and superior mesenteric artery with the existence of peritonitis carcinomatosa (patients 7, 8, 10, and 11, T4 N1 M1). However, all of these patients were of stage III after localization of their tumors during intraoperative diagnosis, including cytology of ascites. Therefore, patients 7, 8, 10, and 11 underwent intraoperative radiation (2500 rad [25 Gy]), whereas patient 6 underwent radical distal pancreatectomy with lymph node dissections including resection of the celiac axis and portal vein. The numbers of Foxp3$^+$CD4$^+$ T cells in these patients were increased compared with the control HD group, although the numbers were rather low among the other inoperable patients whose cancer progressed to advanced forms (existence of peritonitis carcinomatosa or further metastasis, M1, stage IV). These cases were difficult to resect technically, although the cancer was localized in the primary site and not associated with peritonitis carcinomatosa or further metastases. These findings mean that the cancers in these cases are localized biologically, despite the technical differences associated with surgical operations, and suggest that higher numbers of Foxp3$^+$CD4$^+$ T cells indicate higher levels of metastasis.

Furthermore, the populations of Foxp3$^+$CD4$^+$ T cells were significantly higher in the PC group than in patients with endocrine tumors of the pancreas (pathologically benign insulinoma or benign nonfunctional endocrine tumor) (data not shown). This finding strongly suggests that Foxp3$^+$CD4$^+$ T cells are responsible for malignant tumor immunity.

Second, we measured Foxp3 expression at the mRNA level using real-time polymerase chain reaction (data not shown), because it is important to identify accurate numbers of Foxp3$^+$CD4$^+$ T cells for FACScan analyses when considering whether to undertake a radical resection. It is also necessary that further metastases are assessed by more reliable detectors, such as positron emission tomography.[13]

Third, we assessed regT cells as Foxp3$^+$CD4$^+$ T cells (including CD25$^+$ and CD25$^-$ T cells), that is, Foxp3$^+$CD4$^+$ CD25$^+$ T cells and Foxp3$^+$CD4$^+$CD25$^-$ T cells, in the present study. Although CD4$^+$CD25$^-$ T cells were reported to have regulatory activities,[9] further examinations of T cells among the Foxp3$^+$CD4$^+$CD25$^+$ T cells and Foxp3$^+$CD4$^+$ CD25$^-$ T cells are required.

Finally, further experiments should be performed with larger patient numbers to investigate the relationships between the stages of PC and the populations of Foxp3$^+$CD4$^+$CD25$^+$ T cells, that is, to estimate the immune state of PC patients. If such correlations exist, the possibility of modulating the immune system for tumor rejection, for example, by modulating the Foxp3$^+$CD4$^+$CD25$^+$ T-cell population in human peripheral blood, may lead to the development of tumor-specific antitumor immunity in the near future.

REFERENCES

1. Imamura M, Doi R, Imaizumi T, et al. A randomized multicenter trial comparing resection and radiochemotherapy for resectable locally invasive pancreatic cancer. *Surgery*. 2004;136:1003–1011.
2. Ishikawa O, Ohhigashi H, Sasaki Y, et al. Practical usefulness of lymphatic and connective tissue clearance for the carcinoma of the pancreas head. *Ann Surg*. 2000;232:786–795.
3. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25$^+$CD4$^+$ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol*. 1999;163:5211–5218.

4. Ikemoto T, Yasutomo K, Tashiro S, et al. Donor-specific tolerance induced by simultaneous allogeneic islet transplantation with $CD4^+CD25^+$ T-cell into hepatic parenchyma in mice. *J Med Invest.* 2004;51:178–185.
5. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single IL-2 mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* 1995;155:1151.
6. Yamagiwa S, Gray JD, Hashimoto S, et al. A role for TGF-b in the generation and expansion of $CD4^+CD25^+$ regulatory T cells from human peripheral blood. *J Immunol.* 2001;166:7282–7289.
7. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003;299:1057.
8. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing $CD4^+CD25$ (high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol.* 2004;173:1444–1453.
9. Curotto de Lafaille M, Andreia CL, Kutchukhidze N, et al. CD25-T cells generate $CD25^+Foxp3^+$ regulatory T cells by peripheral expansion. *J Immunol.* 2004;173:7259–7268.
10. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 29th WMA General Assembly, Tokyo, Japan, October 1975.
11. Sobin LH, Wittekind LH. *International Union Against Cancer (UICC). TNM Classification of Malignant Tumors.* 6th ed. New York: Willy-Liss, Inc; 2002:87–90.
12. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol.* 2002;169: 2756–2761.
13. Nishiyama Y, Yamamoto Y, Yokoe K, et al. Contribution of whole body FDG-PET to the detection of distant metastasis in pancreatic cancer. *Ann Nucl Med.* 2005;19:491–497.
14. Cheng F, Wang HW, Cuenca A, et al. A critical role for Stat3 signaling in immune tolerance. *Immunity.* 2003;19:425–436.
15. Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory $CD4^+CD25^+$ T cells. *Clin Immunol.* 2005;115:10–18.
16. Albert MH, Liu Y, Anasetti C, et al. Antigen-dependent suppression of alloresponses by Foxp3-induced regulatory T cells in transplantation. *Eur J Immunol.* 2005;35:1–10.
17. Cunningham D, Chau I, Stocken D, et al. Phase III randomised comparison of gemcitabine (GEM) versus gemcitabine plus capecitabine (GEM-CAP) in patients with advanced pancreatic cancer. *Eur J Cancer.* 2005;4:11.
18. Chua YJ, Cunningham D. Chemotherapy for advanced pancreatic cancer. *Best Pract Res Clin Gastroenterol.* 2006;20(2):327–348.

Cancer Gene Therapy (2006) 13, 520–529

www.nature.com/cgt

ORIGINAL ARTICLE

Peritumoral injection of adenovirus vector expressing NK4 combined with gemcitabine treatment suppresses growth and metastasis of human pancreatic cancer cells implanted orthotopically in nude mice and prolongs survival

Y Ogura[1], K Mizumoto[1], E Nagai[1], M Murakami[1], N Inadome[1], M Saimura[1], K Matsumoto[2], T Nakamura[2], M Maemondo[3], T Nukiwa[3] and M Tanaka[1]

[1]*Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan;* [2]*Department of Molecular Regenerative Medicine, Osaka University Graduate School of Medicine, Osaka, Japan and* [3]*Department of Respiratory Oncology and Molecular Medicine, Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan*

NK4 or adenovirus vector expressing NK4 (Ad-NK4) can act bifunctionally as a hepatocyte growth factor antagonist and angiogenesis inhibitor and has potential value in cancer therapy. The aim of this study was to evaluate the therapeutic efficacy of Ad-NK4 in combination with gemcitabine (GEM) against pancreatic cancer. *In vitro* study showed a strong antiproliferative effect of GEM and a potent anti-invasive effect of Ad-NK4 against pancreatic cancer cells. In *in vivo* experiments, SUIT-2 human pancreatic cancer cells were implanted into the pancreas of nude mice. Mice were treated with Ad-NK4 by injection into the peritumoral region of the pancreas on day 5 after implantation followed by weekly i.p. injections of GEM. On day 28 after implantation, pancreatic tumor volume was significantly smaller than that in mice treated with Ad-LacZ, Ad-NK4 alone, or GEM alone. Furthermore, combination therapy completely suppressed peritoneal dissemination and liver metastases, leading to significantly increased survival. Histologic and immunohistochemical assays of primary tumors indicated that combination therapy prohibited both cell proliferation and angiogenesis, resulting in high levels of apoptosis. These results suggest that peritumoral injection of Ad-NK4 plus GEM is a potent combination therapy for pancreatic cancer.
Cancer Gene Therapy (2006) **13,** 520–529. doi:10.1038/sj.cgt.7700921; published online 9 December 2005

Keywords: combination therapy; adenovirus vector; NK4; gemcitabine; pancreatic cancer

Introduction

Pancreatic cancer possesses the potential for strong growth and metastasis and is one of the most fatal human cancers. In Japan, it has been estimated that this disease causes 19 000 deaths per year; the number has increased 2.5-fold in the past 20 years.[1] Despite developments in diagnostic imaging, the majority of patients with pancreatic cancer are diagnosed at the advanced stage of the disease and show an overall 5-year survival rate of only 1–4%.[2] Most patients treated by curative resection develop incurable local relapse, liver metastases, and/or peritoneal dissemination. Conventional adjuvant therapy such as radiation, chemotherapy, immunotherapy, or a combination of these, has little beneficial effect on this aggressive neoplasm. Among the limited chemotherapeutic agents available, gemcitabine (GEM), a new deoxycytidine analogue, has become the first-line treatment for pancreatic cancer.[3] Intracellularly phosphorylated GEM interacts directly with DNA and potently inhibits the proliferation of pancreatic cancer cells. However, the survival benefit of GEM for advanced pancreatic cancer remains limited.

Hepatocyte growth factor (HGF) was identified first as a potent mitogen for hepatocytes,[4,5] however, a distinct role of HGF in cancer development via tumor–stromal interactions has been identified.[6–8] In pancreatic cancer, fibroblast-derived HGF mainly confers mitogenic potential by binding to the c-Met receptor,[9,10] which is frequently overexpressed in this cancer, but displays minimal mitogenic activity.[7,11,12] Phosphorylated c-Met receptor accelerates the migratory and invasive ability of pancreatic cancer via MAP kinase activation.[13,14] Furthermore, increased mRNA and protein levels of c-Met and/or HGF have been frequently reported in

Correspondence: Dr K Mizumoto, Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan.
E-mail: mizumoto@med.kyushu-u.ac.jp.
Received 27 February 2005; revised 23 September 2005; accepted 4 October 2005; published online 9 December 2005

human pancreatic carcinoma in contrast to weak expression in normal tissue.[11,15,16] Additionally, high levels of HGF have been reported in peritoneal fluid after pancreatic cancer surgery, and ascitic fluid strongly stimulates the invasive ability of pancreatic cancer cells.[7] Thus, HGF and c-Met receptor may be involved in the malignant behavior of pancreatic cancer, and prevention of the HGF-c-Met association may be of considerable therapeutic value.

We developed NK4, a synthetic competitive antagonist of HGF, which comprises the N-terminal hairpin domain and subsequent four kringle domains of the HGFα subunit.[6,17,18] NK4 binds competitively to the c-Met receptor and abrogates the accelerated motility induced by HGF. Additionally, NK4 exerts an antiangiogenic effect independent of its activity as an HGF antagonist.[19] NK4 may be of value in cancer therapy by targeting the malignant behavior of cancer cells rather than by killing the cells directly. We reported that, *in vitro*, recombinant NK4 or adenovirus vector-expressing NK4 (Ad-NK4) inhibits spreading and invasion of human pancreatic cancer cells[7] and, *in vivo*, suppresses growth, invasion, and metastasis of human pancreatic cancer cells orthotopically implanted into nude mice.[8] Hence, gene therapy with Ad-NK4 may be an effective cytostatic treatment to prohibit motility and angiogenesis of pancreatic cancer.

Combination treatment with Ad-NK4 plus GEM may provide an optimal therapy for controlling pancreatic cancer. In the present study, we analyzed the therapeutic efficacy of Ad-NK4 in combination with GEM against pancreatic cancer *in vitro* and *in vivo*. *In vitro* results indicated that GEM exerts a strong antiproliferative effect but is relatively ineffective against the invasive ability of pancreatic cancer. In contrast, Ad-NK4 exerted no antiproliferative effect but did show a potent anti-invasive effect. *In vivo* experiments indicated that peritumoral injection of Ad-NK4 combined with weekly GEM treatment significantly reduced growth, angiogenesis, and metastasis of human pancreatic cancer cells orthotopically implanted into nude mice.

Materials and methods

Construction of recombinant adenovirus

A recombinant adenoviral vector expressing human NK4 was constructed as described previously.[20] In brief, the vector was generated by homologous recombination of the pJM17 plasmid[21] and shuttle plasmid vector pSV2+[22] containing an expression cassette with the cytomegalovirus early promoter/enhancer, followed by human NK4 cDNA[17] and a polyadenylation signal. A control vector expressing bacterial β-galactosidase (LacZ) was constructed by the same procedure with pJM17 and pCA17 containing the LacZ gene. Recombinant adenovirus (denoted Ad-NK4 or Ad-LacZ) was propagated in HEK 293 cells and was purified by CsCl gradient centrifugation. Adenovirus titer in plaque-forming units (pfu) was determined by plaque formation assays with HEK 293 cells. The multiplicity of infection (MOI) was defined as the ratio of the total number of pfus used in a particular infection to the total number of cells to be infected.

Cell culture and materials

Human pancreatic cancer cell lines used in this study: PaCa-2 cells provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan), and KP-2, Panc-1, and SUIT-2 cells generously donated by Dr Haruo Iguchi (National Kyushu Cancer Center, Fukuoka, Japan). Cells were cultured in RPMI medium supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), and 10% fetal bovine serum (FBS) at 37°C in a humidified 90% air and 10% CO_2 environment. Total cell numbers were quantified with a particle distribution counter (CDA 500; Sysmex, Kobe, Japan). GEM (2′,2′-difluorodeoxycytidine) was kindly provided by Eli Lilly and Company (Indianapolis, IN). GEM was dissolved in phosphate-buffered saline (PBS) and added to cell cultures or injected peritumorally into nude mice at the indicated concentrations.

Quantitative analysis of c-Met receptor mRNA levels in human pancreatic cancer cell lines by reverse-transcription (RT) PCR

Total RNA was extracted from cultured cells with the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) with DNase I (Roche Diagnostics) treatment according to the manufacturer's instructions. We designed specific primers for c-Met (forward primer, tgatgatgaggtggacaca; reverse primer, ctatggcaaggagcaaaga) and beta-actin (forward primer, aaatctggcaccacaccttc; reverse primer, ggggtgttgaaggtctcaaa), performed BLAST searches to ensure primer specificity, and performed RT-PCR with a QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. In brief, the total volume of the reaction mixture was 20 μl and contained 10 μl 2 × SYBR Green Buffer, 0.2 μl RT Mix, 1 μl each primer (10 μM), and 1 μl total RNA (0.01 μg/μl). The reaction mixture was first incubated at 50°C for 15 min to allow for RT. PCR was then initiated at 95°C for 10 min to activate modified Taq polymerase, followed by a 45-cycle amplification (95°C for 15 s, 55°C for 20 s, and 72°C for 10 s) and one cycle (95°C for 0 s, 65°C for 15 s, and 0.1°C/s to 95°C) for melting analysis. For relative quantification, the expression of the c-Met gene was normalized to that of the beta-actin housekeeping gene and expressed as the ratio of relative expression of c-Met mRNA to that of beta-actin mRNA.

Cell proliferation assay

Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide (PI) as described previously.[23] Pancreatic cancer cells, seeded in 90-mm dishes at a density of 1×10^6 cells per dish, were infected with Ad-NK4 or Ad-LacZ in 6 ml of medium at 37°C for 1 h, after which the medium was replaced. After 24 h, parental cells or Ad-NK4- or Ad-LacZ-infected pancreatic cancer cells were plated at 2×10^4 cells/well in 24-well tissue culture plates (Becton Dickinson Labware,

Bedford, MA) and cultured for 24 h. After determination of initial cell numbers by PI assay, infected or noninfected pancreatic cancer cells were further cultured in the presence or absence of GEM at the indicated concentrations for 72 h. PI (30 μM) and digitonin (600 μM) were added to each well to label all nuclei with PI. Fluorescence intensity, corresponding to total cells, was measured by a CytoFluor II multi-well plate reader (PerSeptive Biosystems Inc., Framingham, MA) with 530-nm excitation and 645-nm emission filters. Cell proliferation was defined as the ratio of fluorescence intensity at this point to that measured at the beginning of the experiment. All experiments were performed in triplicate wells and were repeated at least three times.

Invasion assay

Invasion of pancreatic cancer cells was measured as the number of cells invading through Matrigel-coated transwell chambers (Becton Dickinson, Franklin Lakes, NJ). Transwell inserts with 8-μm pores were coated with Matrigel (20 μg/well, Becton Dickinson Labware). At 24 h after adenovirus infection (Ad-NK4 or Ad-LacZ), parental or adenovirus-infected pancreatic cancer cells suspended in medium containing 10% FBS were added to the inner cup of a Matrigel invasion chamber at a density of 1×10^5 cells/cm^2. GEM was added to the outer cup at concentrations ranging from 1.0 to 100 nM. HGF (10 ng/ml) was added to the medium in the outer cup to accelerate invasion, and the cells were incubated for 24 h. Cells that had degraded the Matrigel and invaded to the lower surface of the Matrigel-coated membrane were fixed with 70% ethanol and stained with hematoxylin and eosin; five randomly selected fields ($\times$400) were then quantified by light microscopy.

Animals and orthotopic implantation of tumor cells

Female athymic nude mice (6 weeks old) (BALBc nu/nu) were obtained from Japan SLC (Hamamatsu, Japan). The mice were housed in laminar-flow cabinets under specific pathogen-free conditions in facilities approved by Kyushu University. SUIT-2 cells were briefly treated with trypsin-EDTA and washed twice with serum-free medium. Cell suspensions of 1×10^6 cells/0.1 ml were injected into the pancreas tail of mice under anesthesia. To prevent leakage of intrapancreatic tumor cells, a cotton swab was held over the injection site for 1 min. All mice tolerated the surgical procedure well.

NK4 expression in various tissues after peritumoral injection of Ad-NK4

To evaluate NK4 expression in abdominal tissues, three mice subjected to peritumoral injection of Ad-NK4 at 5×10^8 pfu/0.2 ml 5 days after orthotopic implantation were killed 5 days after viral injection. Tissue samples of pancreas including implanted tumor, liver, and spleen were homogenized in ice-cold lysis buffer consisting of 1% Triton X-100, 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 0.1% bovine albumin. Supernatants were collected after centrifugation at 12 000 r.p.m. for 10 min. Expression of NK4 was assayed by enzyme-linked immunosorbent assay (ELISA) with a human HGF ELISA kit (Immunis HGF EIA; Institute of Immunology, Tokyo, Japan) according to the manufacturer's protocol.

Treatment with Ad-NK4 in combination with GEM

Ad-LacZ or Ad-NK4 at 5×10^8 pfu/0.2 ml was injected into the peritumoral region of the pancreas 5 days after orthotopic implantation. At 2 days after injection of Ad-LacZ or Ad-NK4, mice were treated weekly with i.p. administration of GEM or PBS for 3 weeks. All mice were killed when one mouse became moribund at approximately 4 weeks, and body weights were determined. Pancreas were excised and weighed. The volume of primary pancreatic tumors and the incidence of peritoneal dissemination and liver metastasis were evaluated. Pancreatic tumor diameter was measured with calipers. Pancreatic tumor volume was then calculated according to the following formula: tumor volume $= ab^2/2$, where a is the longest diameter of the tumor, and b is the shortest diameter. Tissue samples of the pancreatic tumors were subjected to histologic and immunohistochemical analyses. In another experiment, 40 mice (10 per group) were analyzed with respect to survival.

Immunohistochemical staining and cell counting

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

Statistical analysis

Statistical significance was evaluated by Mann–Whitney U nonparametric test. $P<0.05$ based on a two-tailed test was considered to indicate statistical significance. Survival was analyzed by log-rank analysis of Kaplan–Meier curves.

Results

c-Met receptor mRNA levels in pancreatic cancer cell lines

c-Met receptor mRNA expression was measured in four pancreatic cancer cell lines by RT-PCR. All cell lines

expressed c-Met mRNA (data not shown). SUIT-2 cells expressed a high level of c-Met. Panc-1 and KP-2 cells showed moderate expression. PaCa-2 cells showed very low expression.

Antiproliferative effect of Ad-NK4 and/or GEM in vitro

We first determined the effect of Ad-NK4 or GEM on cell proliferation *in vitro* with SUIT-2 cells expressing a high level of c-Met mRNA (Figure 1). Untreated SUIT-2 cell number increased 9–11-fold in 72 h. Infection with Ad-NK4 or Ad-LacZ did not show any antiproliferative effect at MOIs ranging from 1 to 100 (Figure 1a). In contrast, proliferation was inhibited by GEM in a concentration-dependent manner, and 100 nM GEM completely inhibited proliferation (Figure 1b). Simultaneous treatment with Ad-NK4 or Ad-LacZ at 50 MOI plus 100 nM GEM did not show any additive effect on cell proliferation inhibition induced by GEM, indicating that the antiproliferative effect was induced by GEM alone. Similar results were obtained with other cell lines; Ad-NK4 did not show any antiproliferation effect, but GEM inhibited the growth of Panc-1, KP-2, and PaCa-2 cells (Figure 1c).

Inhibition of cell invasion by Ad-NK4 and/or GEM

To investigate the effect of Ad-NK4 and/or GEM on cell invasion, Ad-NK4- or Ad-LacZ-infected SUIT-2 cells or parental cells were cultured in Matrigel invasion chambers in the absence or presence of GEM at concentrations ranging from 1 to 100 nM (Figure 2). The number of invading cells was not affected by infection with Ad-LacZ. In the absence of pretreatment with Ad-NK4, the number of SUIT-2 cells penetrating the Matrigel membrane was moderately decreased by GEM in a concentration-dependent manner (Figure 2a), and 100 nM GEM inhibited invasion by 50% (Figure 2b). The decreased number of invading cells appeared to parallel the decreased cell proliferation induced by GEM. However, it is noteworthy that 50% of the cells maintained invasive capacity after exposure to 100 nM GEM, a concentration that completely inhibited cell proliferation. The number of invading SUIT-2 cells was decreased by 50% in response to infection with Ad-NK4 at 50 MOI. In the presence of GEM, invading Ad-NK4-infected cells decreased in a concentration-dependent manner, and treatment with Ad-NK4 plus 100 nM GEM inhibited invasion by 90%. The effects of Ad-NK4 and/or GEM on cell invasion were also examined in the other cell lines; GEM showed very minor effects (Figure 2c). In KP-2 and Panc1 cells, which showed moderate c-Met mRNA expression. Ad-NK4 inhibited invasion by 44 and 70%, respectively. Ad-NK4 plus GEM inhibited invasion by 74 and 78%, respectively. In contrast, invasion of PaCa-2 cells, which showed low c-Met mRNA expression, were not inhibited by Ad-NK4. Combined treatment with Ad-NK4 and GEM showed the strongest inhibitory effects on invasion in all three cell lines. These *in vitro* results indicate that only combined treatment with Ad-NK4 plus GEM inhibits both cell proliferation and cell invasion.

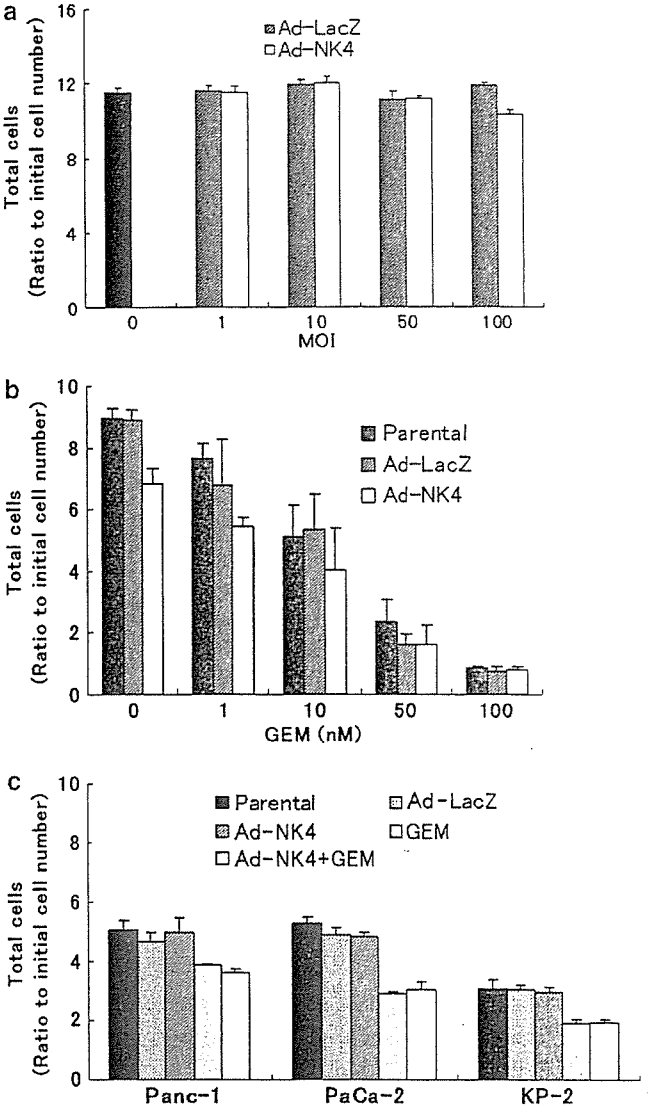


Figure 1 Antiproliferative effect of Ad-NK4 and/or gemcitabine. (a) Cell proliferation of SUIT-2 cells treated with Ad-NK4 or Ad-LacZ. SUIT-2 cells were infected with Ad-NK4 or Ad-LacZ at MOIs ranging from 1 to 100 in medium at 37°C for 1 h. Infected SUIT-2 cells were plated at 2×10^4 cells/well in 24-well tissue culture plates and cultured for 24 h. After further incubation for 72 h, cell proliferation of infected and non-infected SUIT-2 cells was evaluated by propidium-iodide assay as described in Materials and methods. Ad-NK4 showed no antiproliferative effect. (b) Cell proliferation of SUIT-2 cells treated with Ad-NK4 or Ad-LacZ and/or GEM. SUIT-2 cells were infected with Ad-NK4 or Ad-LacZ at an MOI of 50 in medium at 37°C for 1 h. As described in (a), cell proliferation of infected and non-infected SUIT-2 cells in the absence or presence of GEM at the concentrations of 1–100 nM for 72 h was evaluated. A strong antiproliferative effect was induced by GEM in a concentration-dependent manner, but no additive effect was observed by Ad-NK4. (c) Cell proliferation of Panc-1, PaCa-2, and KP-2 cells treated with Ad-NK4 or Ad-LacZ at 50 MOI and/or 100 nM GEM. Ad-NK4 showed no antiproliferative effect, but GEM inhibited proliferation of Panc-1, PaCa-2, and KP-2 cells. Values represent the mean ± s.d. of triplicate measurements.

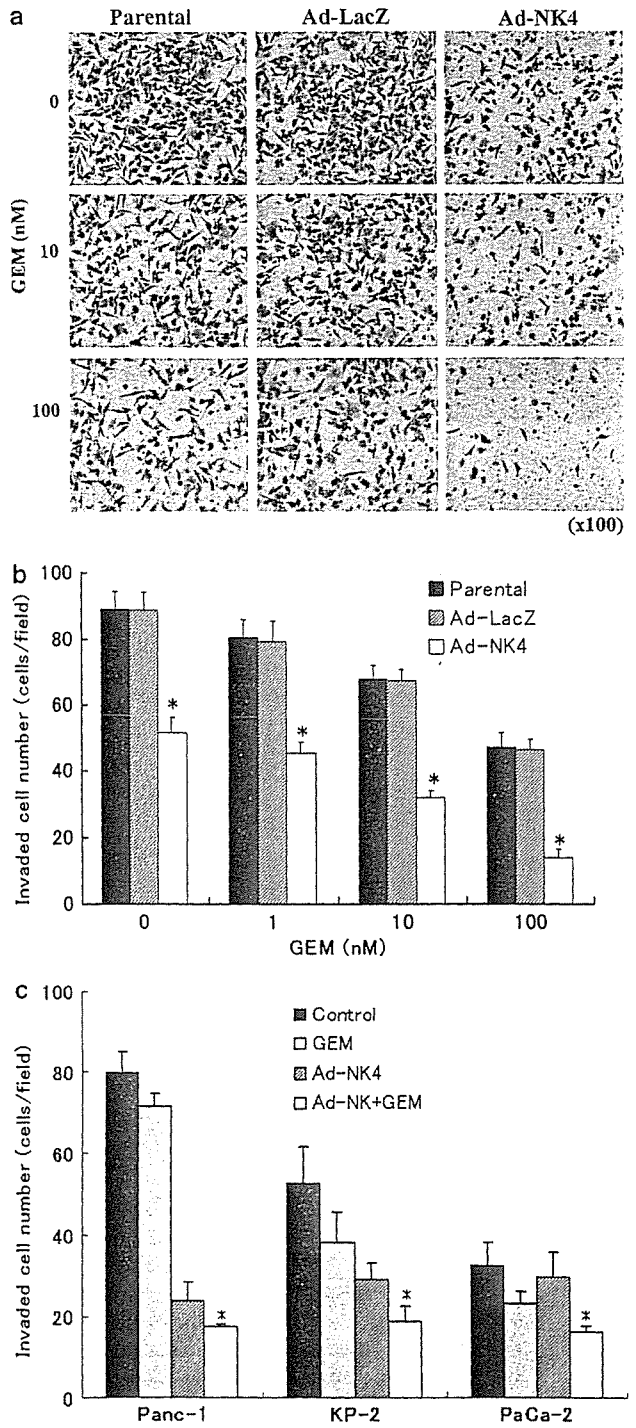


Figure 2 Inhibition of invasion by Ad-NK4 alone or in combination with GEM. (a) Appearance and number of SUIT-2 cells invading through the Matrigel and filter membrane. SUIT-2 cells infected with Ad-NK4 or Ad-LacZ or uninfected cells (Parental) were seeded on Matrigel and cultured in the absence or presence of GEM at concentrations ranging from 1.0 to 100 nM, in addition to HGF (10 ng/ml), in the outer cup. (b) Ad-NK4 significantly suppressed invasion compared to Ad-LacZ at every concentration of GEM (*$P<0.0001$). (c) Inhibition of cell invasion by treatment with 50 MOI Ad-NK4 and/or 100 nM GEM in Panc-1, KP-2, and PaCa-2 cells. Ad-NK4 plus GEM significantly suppressed invasion compared to control in all of the cell lines (*$P<0.001$). Values represent the mean ± s.d. of triplicate measurements.

NK4 expression in nude mice after peritumoral injection of Ad-NK4

Peritumoral injection of Ad-NK4 induced extremely high expression of NK4 in the pancreas of nude mice. NK4 expression in the pancreas and including the implanted tumor was $63.5 \pm 18.6\ \mu g/g$ protein 5 days after viral injection, but NK4 expression in the liver or spleen was undetectable by ELISA. These results indicate that sufficient transduction of NK4 was introduced only in the peritumoral region of the pancreas.

Inhibition of growth and metastasis of human pancreatic carcinoma implanted into the pancreas of athymic nude mice

The therapeutic efficacy of Ad-NK4 plus GEM was confirmed in an orthotopic model of pancreatic cancer in athymic nude mice. First, the therapeutic effect of GEM alone was evaluated. At 7 days after implantation of tumor cells, six mice per group were treated weekly with 20, 40, 80, 160, or 320 mg/kg GEM by i.p. administration for 3 weeks, resulting in 30, 58, 75, 75, and 82% growth inhibition of SUIT-2 tumors, respectively, compared to that of untreated mice 4 weeks after implantation. Because $\geqslant$80 mg/kg GEM showed the same degree of growth inhibition, 80 mg/kg concentration was used exclusively thereafter. Next, mice were randomly assigned to treatment groups ($n = 10$ each). Mice in the Ad-NK4 group were treated with Ad-NK4 by injection into the peritumoral region of the pancreas followed by weekly i.p. injection of PBS for 3 weeks. Mice in the GEM group were treated with Ad-LacZ followed by weekly i.p. injection of GEM. Mice in the Ad-NK4 plus GEM group were treated with Ad-NK4 followed by weekly i.p. injection of GEM. Control mice were treated with Ad-LacZ followed by weekly i.p. injection of PBS. On day 28 after implantation, macroscopic identification of pancreatic tumors was confirmed in all groups (Figure 3), and peritoneal dissemination was also noted in the retroperitoneum, mesenterium, and hepatic hilum in control mice. Liver metastasis was found in 40% of control mice. No treatment produced cumulative toxicity or a change in body weight (Table 1). Pancreatic tumor volume was significantly lower in mice treated with Ad-NK4 or GEM than in control mice (Figure 4). Moreover, tumors in mice treated with Ad-NK4 plus GEM were significantly smaller than those treated with Ad-NK4 alone ($P<0.001$) or GEM ($P<0.001$) alone. The incidence of peritoneal dissemination was decreased by treatment with Ad-NK4 or GEM, and combination therapy completely suppressed peritoneal dissemination (Figure 3b, Table 1). No macroscopic liver metastases were noted in mice treated with Ad-NK4, GEM, or both (Table 1).

Histologic and immunohistochemical analyses

Histologically, primary tumors in all groups were moderately to poorly differentiated adenocarcinomas. Necrotic lesions were identified in the center of the tumor region in mice treated with GEM, Ad-NK4, or both. Tumor cell proliferation was evaluated as the percentage of PCNA-positive cells, and a significant inhibition was

observed in tumors from mice treated with GEM (30%) or GEM plus Ad-NK4 (20%) compared to the percentage in the control group (83%). No significant difference in the number of PCNA-positive cells in tumors was identified between the Ad-NK4 and control groups (Figure 5a and b). Blood vessels in the tumor tissues, as evaluated by staining with anti-CD31 antibody, showed that MVD was significantly reduced in tumors treated with Ad-NK4 (10.5/hpf) or Ad-NK4 plus GEM (9.6/hpf) compared to MVD in the control group (24.4/hpf). No significant difference in tumor MVD was identified between GEM-treated and control mice (Figure 5a and c). The apoptotic index, as determined by the percentage of TUNEL-positive cells, was significantly increased by treatment with Ad-NK4 or GEM, and the highest index was observed in the combination therapy group (Figure 5a and d). These results indicate that GEM suppressed proliferation of tumor cells and that Ad-NK4 inhibited tumor angiogenesis. Combination therapy prohibited both cell proliferation and angiogenesis and showed the highest level of apoptosis.

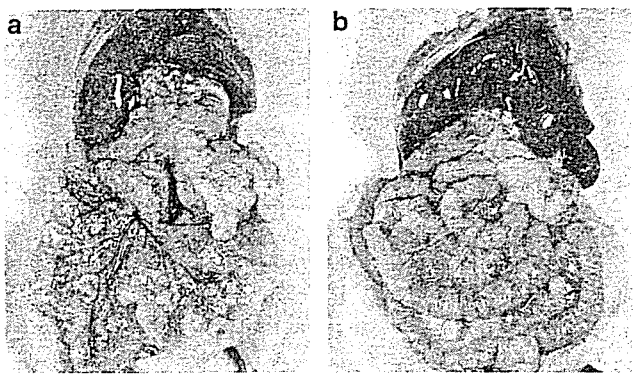


Figure 3 Pancreatic tumors orthotopically implanted into nude mice and treated with Ad-NK4 and GEM. Macroscopic appearance of pancreatic tumor and peritoneal dissemination after (**a**) treatment with Lac-Z and PBS (control) or (**b**) treatment with Ad-NK4 plus GEM. Pancreatic tumors were smaller, and no disseminated nodules were observed in mice treated with Ad-NK4 plus GEM.

Prolonged survival

A final experiment was carried out to determine the effect of combination therapy on survival (Figure 6). Severe cancer cachexia developed in control mice approximately 4 weeks after implantation. The median survival time in the control group was 41 days. The median survival times in the GEM group (50 days) and the Ad-NK4 group (51 days) were similar, differing significantly from that of the control group (GEM versus control, $P<0.05$; Ad-NK4 versus control, $P<0.02$). The median survival time of

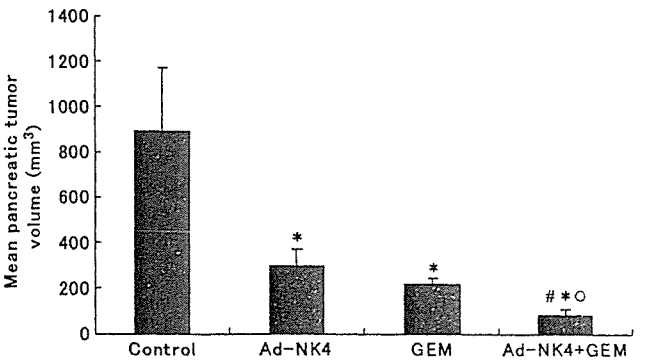
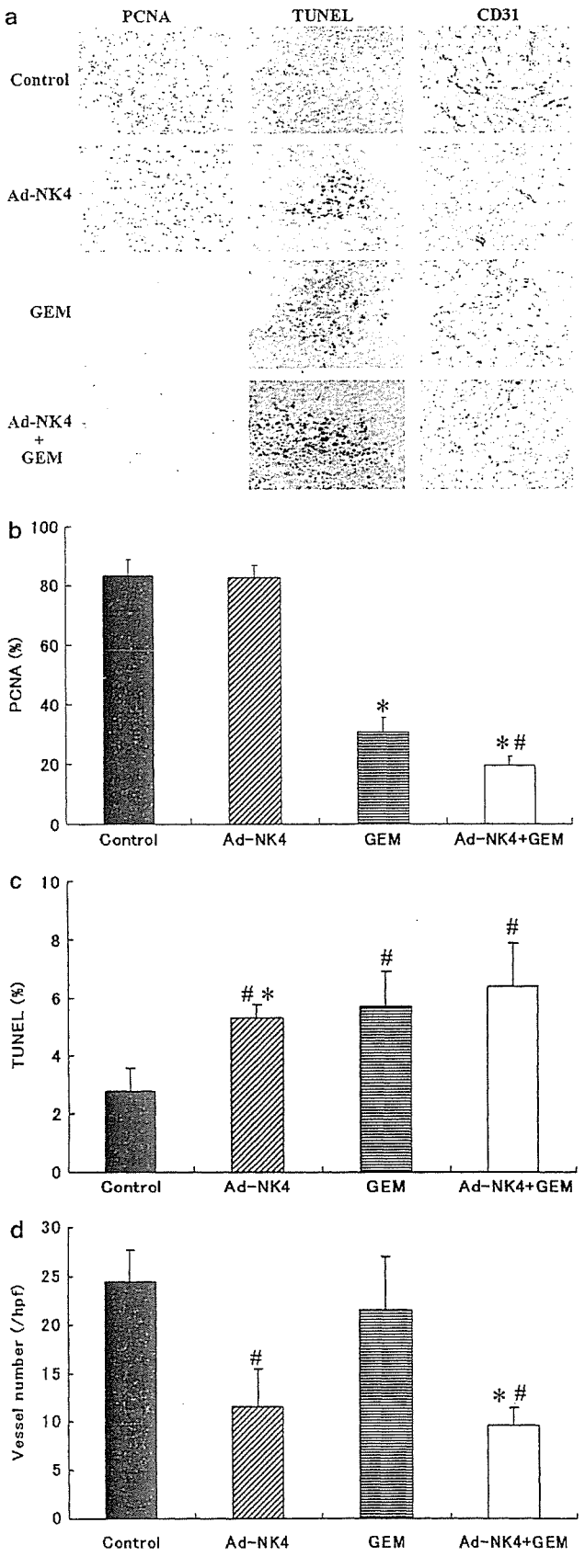


Figure 4 Therapeutic effect of Ad-NK4 plus GEM on primary pancreatic tumor volume of orthotopic human pancreatic cancers in nude mice. Therapy with NK4 alone or GEM alone significantly reduced mean primary pancreatic tumor volume compared to volume of control tumors. Combination therapy (Ad-NK4 plus GEM) significantly decreased mean pancreatic tumor volume compared to volume of control tumors and volume of tumors treated with Ad-NK4 or GEM alone. Bars represent mean±s.d. GEM, Ad-NK4, and Ad-NK4 plus GEM versus control (*$P<0.0001$); GEM versus Ad-NK4 plus GEM (#$P<0.0001$); Ad-NK4 versus Ad-NK4 plus GEM (○$P<0.0001$).

Table 1 Therapies for human pancreatic cancers implanted orthotopically in nude mice

Therapy[a]	*Incidence of macroscopic tumors*			*Total pancreas weight: mg (s.d.)*	*Body weight: g (s.d.)*
	Pancreas tumor[b]	*Liver metastasis*[c]	*Peritoneal dissemination*[d]		
Control	10/10	4/10	10/10	823 (±146.39)	20.994 (±2.178)
GEM	10/10	0/10	4/10	449 (±48.87)	21.793 (±1.257)
Ad-NK4	10/10	0/10	3/10	563 (±112.35)	21.308 (±1.097)
Ad-NK4+GEM	10/10	0/10	0/10[e]	334[f] (±50.51)	21.115 (±0.835)

[a]SUIT-2 human pancreatic cancer cells (1×10^6) were injected into the pancreas tail of nude mice. Groups of mice were treated according to following: GEM group: peritumoral injection of Ad-LacZ (5×10^8 pfu) 5 days after cell injection and i.p. injection of GEM (80 mg/kg) weekly starting 7 days later cell injection Ad-NK4 group: perifumoral injection of Ad-NK4 (5×10^8 pfu) 5 days after cell injection and i.p. injection of PBS weekly starting 7 days after cell injection. Ad-NK4 plus GEM group: (same as above but combined). Control group: Ad-LacZ injection and i.p. injection of PBS. All mice were killed at 4 weeks.
[b]Number of positive mice per number of mice receiving injections.
[c]Number of mice with visible nodules (>1 mm in diameter) per number of mice receiving injections.
[d]Number of mice with enlarged disseminated nodules per number of mice receiving injections.
[e]$P<0.05$ versus Control.
[f]$P<0.0001$ versus Control, GEM, or Ad-NK4.



mice treated with Ad-NK4 plus GEM was 66 days (versus GEM alone, $P<0.01$; versus Ad-NK4, $P=0.031$; versus control, $P<0.001$).

Discussion

We showed that peritumoral injection of Ad-NK4 in combination with GEM treatment strongly inhibited growth, peritoneal dissemination, and liver metastasis of human pancreatic cancer cells implanted into the pancreas of athymic nude mice and prolonged their survival.

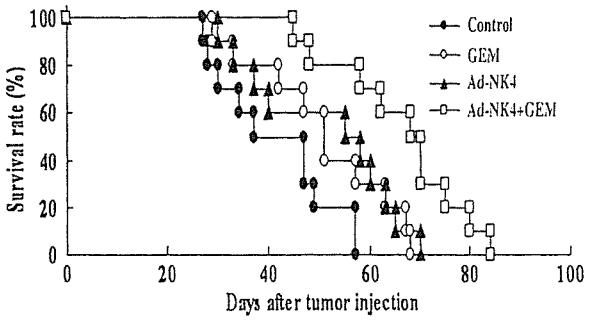


Figure 6 Survival of mice in each treatment group, analyzed by Kaplan–Meier's method and compared by log-rank test ($n=10$). Control, ●; GEM alone, ○ ($P<0.05$ versus control); Ad-NK4 alone, ▲ ($P<0.02$ versus control); Ad-NK4 plus GEM, □ ($P<0.001$ versus control). Mice treated with Ad-NK4 plus GEM showed longer survival than mice treated with each agent alone, ($P<0.01$ for GEM; $P=0.031$ for Ad-NK4).

Figure 5 (a) Immunohistochemical staining of primary pancreatic tumors growing orthotopically in nude mice for cell proliferation (PCNA), cell death (TUNEL), and angiogenesis (CD31) after treatment with Ad-NK4, GEM, or Ad-NK4 plus GEM. (b) PCNA-positive cells were counted in 10 microscopic fields per tumor section at × 100. Total number of cells/field and number of PCNA-positive cells in each field were counted to calculate median percentage in each tumor section. Proliferation index was significantly less in tumors treated with gemcitabine or with Ad-NK4 plus GEM compared to tumors in controls ($^{*}P<0.0001$). Difference between GEM and Ad-NK4 plus GEM treatment was significant ($^{\#}P<0.0001$). Bars represent mean ± s.d. (c) TUNEL-positive cells were counted in 10 microscopic fields per tumor section at × 100. Total number of cells/field and number of TUNEL-positive cells in each field were counted to calculate median percentage in each tumor section. Apoptotic index, as determined by TUNEL, was significantly greater in tumors treated with GEM alone, with Ad-NK4 alone, or with Ad-NK4 plus GEM compared to that of controls ($^{*}P<0.0001$). Difference between GEM and Ad-NK4 treatment was not significant ($^{*}P=0.7$). Bars represent mean ± s.d. (d) Mean number of blood vessels per high-power field (hpf) was significantly less in tumors treated with Ad-NK4 or with Ad-NK4 plus GEM compared to those of control tumors or tumors treated with GEM alone. Difference between Ad-NK4 and combination therapy was not significant ($^{*}P=0.26$). Bars represent mean ± s.d. NK4 alone and combination therapy versus GEM alone, $^{\#}P<0.0001$.