Chemotherapeutic agents potentiate adenoviral gene therapy for pancreatic cancer

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Adenovirus-mediated gene therapy combined with chemotherapeutic agents is expected to represent a new approach for treating pancreatic cancer. However, there have been no reports of definitive effects of chemotherapeutic agents on adenovirus-mediated gene therapies. In the present study, we investigated the effects of chemotherapeutic agents on the transduction efficiency of an adenovirus-based gene therapy. Adenovirus (Ad-NK4) expressing NK4, which acts as a hepatocyte growth factor antagonist, was used as a representative gene therapy. Pancreatic cancer cells infected with Ad-NK4 were treated with chemotherapeutic agents (5-fluorouracil [5FU], cisplatin or etoposide), and the NK4 levels in their culture media were measured. To examine the effects of chemotherapeutic agents *in vivo*, Ad-NK4 was administered to subcutaneous tumors in mice after treatment with the agents, and the tumor NK4 levels were measured. The NK4 levels in culture media from cells treated with 5FU, cisplatin and etoposide were 5.2-fold ($P = 0.026$), 6-fold ($P < 0.001$) and 4.3-fold ($P < 0.001$) higher than those of untreated cells, respectively. The chemotherapeutic agents also increased Ad-NK4 uptake. The NK4 levels in tumors treated with 5FU, cisplatin and etoposide were 5.4-fold ($P = 0.006$), 11.8-fold ($P < 0.001$) and 4.9-fold ($P = 0.017$) higher than those in untreated tumors, respectively. The present findings suggest that chemotherapeutic agents significantly improve the efficiency of adenovirus-mediated gene transfer in pancreatic cancer. Furthermore, they will contribute to decreases in the adenovirus doses required for gene transfer, thereby controlling the side-effects of adenovirus infection in normal tissues. (*Cancer Sci* 2009; 100: 722–729)

On the basis of recent advances in our understanding of the molecular biology of a variety of cancers[1–3] molecular therapies that target tumor-specific pathways and interfere with key regulatory cellular functions, such as cancer cell proliferation, differentiation, metastasis and survival, have been extensively studied.[4,5] Many researchers have used monoclonal antibodies, specific antagonists or specific small-molecule inhibitors as antitumor agents against cancer-associated genes. However, monoclonal antibodies are expensive and small-molecule inhibitors have low specificity. These agents also induce allergic reactions, such as skin rashes.[4] The use of viral vectors, which have high gene transfer efficiencies, is one molecular therapy approach that is useful for expressing an antagonist of a target protein. Adenovirus-based vectors are often used owing to their high transduction efficiency and high levels of transient expression of the transfected gene.[6]

Pancreatic cancer is a leading cause of cancer-related death in industrialized countries.[7,8] Most patients with pancreatic cancer have poor outcomes because early diagnosis is difficult and conventional therapies have limited effectiveness.[9] Recently, advances in our understanding of the genetics and epigenetics of pancreatic cancer have revealed that alterations in several tumor-related genes, such as *K-ras*, *p53*, *MMP*, *HGF* and *EGFR*[10–15] may underlie the aggressiveness of this neoplasm and its resistance to conventional therapies.[5] Therefore, molecular therapies for pancreatic cancer are promising new approaches for treating this often fatal disease. Previous studies have used adenovirus-mediated gene transfer to treat pancreatic cancer and have shown that adenovirus-mediated gene therapy can inhibit the progression of pancreatic cancer *in vivo* and *in vitro*.[16,17] However, clinical trials have shown that it is difficult to eradicate pancreatic tumors with adenovirus-mediated gene therapy alone.[18,19] There is also a concern that the adenovirus doses necessary to achieve therapeutic effectiveness may be associated with significant toxicity. Therefore, adenovirus administration alone may not be an effective treatment for pancreatic cancer, and it may be necessary to combine adenovirus-mediated gene therapies with conventional treatments to maximize the antitumor effects of such therapies for pancreatic cancer.

Recently, combinations of chemotherapy and adenovirus-mediated gene therapy have been reported to be effective for cancer treatment. Topf *et al*.[20] reported that administration of an adenoviral vector expressing the cytosine deaminase gene with 5-fluorocytosine suppressed the growth of colon cancer. Similarly, Shieh *et al*.[21] reported that low-dose etoposide enhanced telomerase-dependent adenovirus-mediated cytosine deaminase gene therapy for bladder cancer, and Lee *et al*.[22] reported that adenovirus-I_{κ} B_{α} transduction restored the chemosensitivity of lung cancers showing resistance to cisplatin. These reports suggest that synergistic effects occur with such combination therapies. Furthermore, chemotherapeutic agents such as 5-fluorouracil (5FU) or S-1, cisplatin and etoposide have been used as second-line chemotherapies for pancreatic cancer.[23–27]

Accumulating evidences have shown that hepatocyte growth factor (HGF) accelerates the invasion of pancreatic cancer cells.[12,28,29] Previously, we reported that gene therapy with an adenovirus vector (Ad-NK4) expressing NK4, which acts as an HGF antagonist, showed significant inhibitory effects on the invasion of pancreatic cancer cells.[30–32] In the present study, we investigated the effects of three chemotherapeutic agents, cisplatin, etoposide, and 5FU instead of S-1 that is an oral anticancer drug based on biochemical modulation of 5FU[33,34] which are accepted as promising drugs for the treatment of pancreatic cancer[23–27] on the efficiency of transfer and expression of a target gene. To achieve this, we examined their effects on NK4 expression by Ad-NK4 as a representative gene therapy, and found that all three agents enhanced adenoviral gene transfer *in vitro* and *in vivo*.

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Materials and methods

Cells and reagents. The human pancreatic cancer cell lines SUIT-2, AsPC-1, KP-1 N and KP-2 were generously donated by Dr H. Iguchi (National Shikoku Cancer Center, Matsuyama, Japan) and cultured as described previously.[32] Human recombinant HGF was purified as described previously.[35,36] 5FU was kindly provided by Kyowa Hakko Kogyo Company (Tokyo, Japan), while cisplatin and etoposide were both donated by Nippon Kayaku Company (Tokyo, Japan).

Construction of recombinant adenoviruses. Adenovirus (Ad)-NK4 was constructed as described previously.[37–40] A control vector expressing the bacterial *β-galactosidase* (*β-gal*) gene (*lacZ*) was constructed by the same procedure. Recombinant Ad-NK4 and Ad-lacZ were propagated in HEK293 cells.

Adenovirus infection of cells. Cells were infected with Ad-NK4 or Ad-lacZ at a multiplicity of infection (MOI) of 10 or 50 as described previously.[32] The culture medium was replaced with fresh medium at 1.5 h after the transfection.

Extraction of proteins from cells infected with Ad-NK4. SUIT-2 cells were treated with chemotherapeutic agents and infected with Ad-NK4 as described above. At 2 days after the infection, the cells were lyzed in 500 μL of ice-cold lysis buffer.[32] The supernatants were collected, and adjusted to 1.0 mg/mL.

NK4 expression by Ad-NK4-infected cancer cells. NK4 levels were measured 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.

Invasion assay. The invasiveness of pancreatic cancer cells was quantified as the number of cells invading through Matrigel-coated transwell inserts (Becton Dickinson, Franklin Lakes, NJ, US) as described previously.[41] SUIT-2 cells$^{(1)} \times 10^{7}$ were left untreated or treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM) for 24 h. After removing culture media containing these agents, the cells were infected with Ad-lacZ or Ad-NK4 at an MOI of 50, and the culture media were collected on postinfection day 3. New SUIT-2 cells were seeded in 24-well plates at a density of 1×10^5 cells/cm^2 in the upper chamber and cultured with 750 μL of conditioned media from the SUIT-2 cells treated with the chemotherapeutic agents or left untreated and infected with Ad-lacZ or Ad-NK4. After 72 h of incubation in the presence of 0.01 ng/mL HGF, cells that had invaded to the lower surface of each Matrigel-coated membrane were fixed with 70% ethanol, stained with hematoxylin and eosin (H&E), and counted in five randomly selected fields under a light microscope.

Cell proliferation assay. Cell proliferation was evaluated based on the fluorescence intensity of propidium iodide (PI), as described previously.[42] All experiments were performed in triplicate wells.

Assessment of transgene distribution by evaluation of β-gal expression. At 48 h after Ad-lacZ infection, β-gal activity was detected as described previously.[32]

Real-time polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) assays. The Ad-lacZ DNA contents in infected cells were determined by real-time PCR analysis as described previously[43] with primers for the *β-gal* gene (5′-CACGGCAGAT ACACTTGCTG-3′ and 3′-ATCGCCATTTGACCACTACC-5′).[44] The copy numbers of the viral DNA were calculated from a standard curve of the purified adenovirus vector (cytomegalo virus (CMV)-β-gal) and further adjusted relative to the protein concentration of each lysate. The *dynamin 2*, *coxsackie virus and adenovirus receptor* (*CAR*) and *β3-integrin* mRNA levels were quantified from 100 ng of total RNA by real-time RT-PCR amplification with a QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, US) and primers specific for *dynamin 2* (5′-AGGAGT-ACTGGTTTGTGCTGACTG-3′ and 3′-GTGCATGATGGTCTT-TGGCATGAG-5′)[44] *CAR* (5′-GGCGCTCCTGCTGTGC-3′ and 3′-CTTCTCTACTAACTTTTTCGGTTTC-5′) and *β3-integrin* (5′-GAGGATGACTGTGTCGTCAG-3′ and 3′-AAACTCCTTC-TTGCGCGGTC-5′). The *dynamin 2*, *CAR* and *β3-integrin* mRNA levels were normalized to the corresponding levels of *18S rRNA* amplified with specific primers (5′-GTAACCCGTTGAACC-CCATT-3′ and 3′-GCGATGATGGCTAACCTACC-5′)[45] and expressed as ratios compared with untreated controls.

Electroporation. pcDNA3-NK4 (2.5 μg, NK4-expressing plasmid) or pcDNA3 (2.5 μg, empty vector) was mixed with 5×10^6 SUIT-2 cells and electroporated with a Nucleofector (Amaxa Biosystems GmbH, Koln, Germany) according to the manufacturer's instructions.

Evaluation of chemotherapeutic agent-induced NK4 expression *in vivo* in xenografts in *severe combined immunodeficient (SCID)* mice. To investigate the chemotherapeutic agent-enhanced NK4 expression induced by Ad-NK4 *in vivo*, subcutaneous tumors were established in SCID mice by injection of 5×10^6 SUIT-2 cells into back and both flanks. After 7 days, two mice were, respectively, treated by intraperitoneal (i.p.) administration of 5FU (10 mg/kg), cisplatin (5 mg/kg) or etoposide (10 mg/kg) and two mice were left untreated as controls. At 24 h after the treatment, 5×10^7 plaque forming unit (pfu) of Ad-NK4 (100 μL) was injected into the tumors. To examine the expression levels of NK4 protein in the subcutaneous tumors, the mice were killed at 48 h after the administration of Ad-NK4 and the tumors were excised. Each tumor was homogenized in 300 μL of protein lysis buffer.[32] The protein concentrations in the tumors were adjusted to 10.0 mg/mL with lysis buffer. The NK4 concentrations in each extract were analyzed by ELISA (Immunis HGF EIA).

Statistical analysis. Values are expressed as the mean ± standard deviation (SD). All differences among sets of two groups were analyzed by Student's *t*-test. The level of statistical significance was set at $P < 0.05$. To confirm the induction results, the experiments were repeated at least three times.

Results

Effects of 5FU, cisplatin and etoposide on the expression of a target gene delivered by an adenoviral vector. To investigate the effects of 5FU, cisplatin and etoposide on the expression of a target gene delivered by an adenoviral vector, we measured the NK4 levels in culture media from pancreatic cancer cells infected with Ad-NK4 with or without treatment with these agents. SUIT-2 cells$^{(2)} \times 10^{5}$ were treated with 5FU (2, 5 or 10 μM), cisplatin (1, 2 or 5 μM) or etoposide (2, 5 or 10 μM) for 24 h, and then infected with Ad-NK4 at an MOI of 10. Culture media were collected on postinfection days 1, 2 and 3. NK4 expression by Ad-NK4-infected cells peaked on day 2 after the transfection (data not shown). As shown in Fig. 1(A) and 5U, cisplatin and etoposide increased the NK4 expression levels at day 2 in dose-dependent manners ($P < 0.05$). NK4 expression was not detected in cells that were not infected with Ad-NK4 (data not shown). Furthermore, AsPC-1, KP-1 N and KP-2 cells (2×10^5 cells for each cell line) were treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM) and infected with Ad-NK4 as described above. As shown in Fig. 1(B), pretreatment of these three cell lines with all three agents increased adenovirus-induced NK4 expression, consistent with the results for SUIT-2 cells. To investigate the intracellular NK4 protein levels, we extracted proteins from SUIT-2 cells treated with the three agents and infected with Ad-NK4, and measured the levels of NK4. As shown in Fig. 1(C) and 5U and cisplatin significantly increased the intracellular NK4 protein levels in dose-dependent manners, while etoposide tended to be associated with increased intracellular NK4 protein levels ($P = 0.124$). These data suggest that 5FU, cisplatin and etoposide can enhance the expression of a target gene delivered by an adenovirus vector.

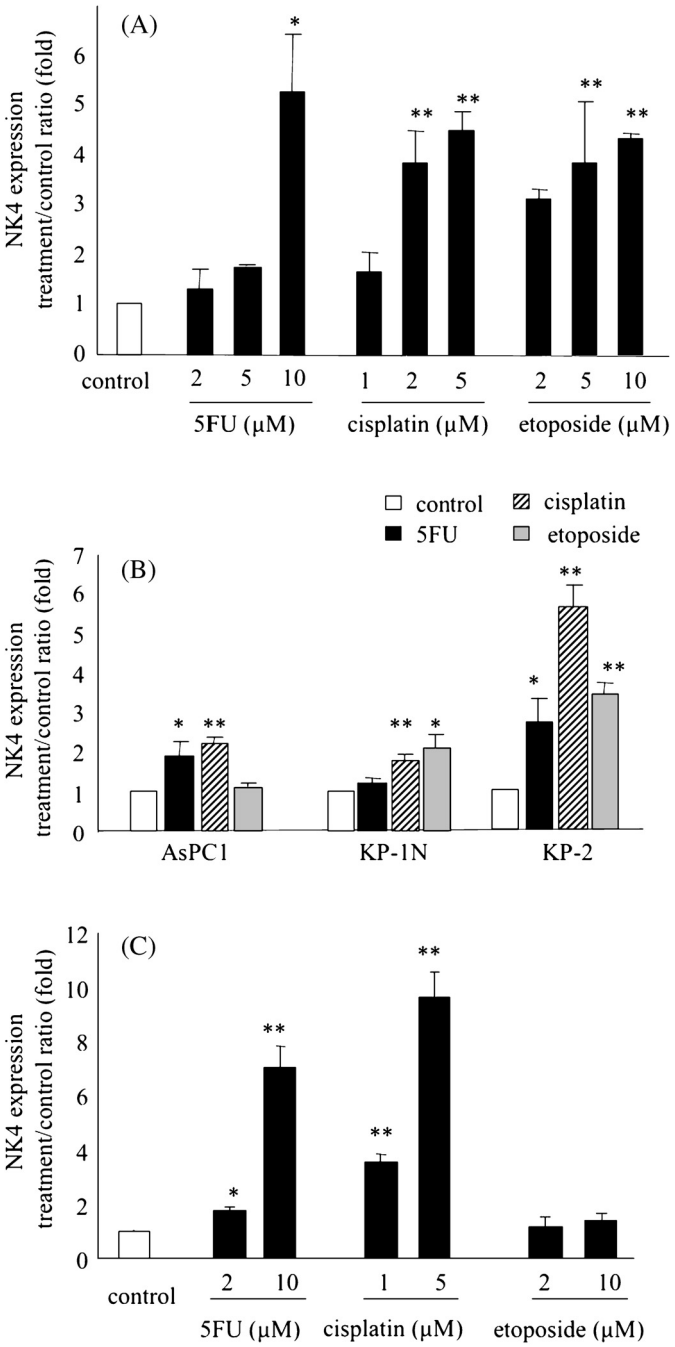

Fig. 1. 5-fluorouracil (5FU), cisplatin and etoposide significantly increase NK4 expression in Ad-NK4-infected cells. (A) SUIT-2 cells were treated with 5FU, cisplatin or etoposide for 24 h and then infected with Ad-NK4 at a multiplicity of infection (MOI) of 10. The NK4 levels in the culture media were measured by enzyme-linked immunosorbent assay (ELISA) on postinfection day 2. Each value represents the mean ± SD of three independent samples. $^{**}P < 0.01$. $^{*}P < 0.05$, compared with control cells. (B) AsPC-1, KP-1 N and KP-2 cells were treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM) and then infected with Ad-NK4 as described in (A). The NK4 levels in the culture media were measured by ELISA on postinfection day 2. $^{**}P < 0.01$. $^{*}P < 0.05$, compared with each control cells. (C), Proteins were isolated from SUIT-2 cells treated with 5FU, cisplatin or etoposide and infected with Ad-NK4 as described above on postinfection day 2, and the NK4 concentrations were determined by ELISA. Each value represents the mean ± SD of three independent samples. $^{**}P < 0.01$. $^{*}P < 0.05$, compared with control cells.

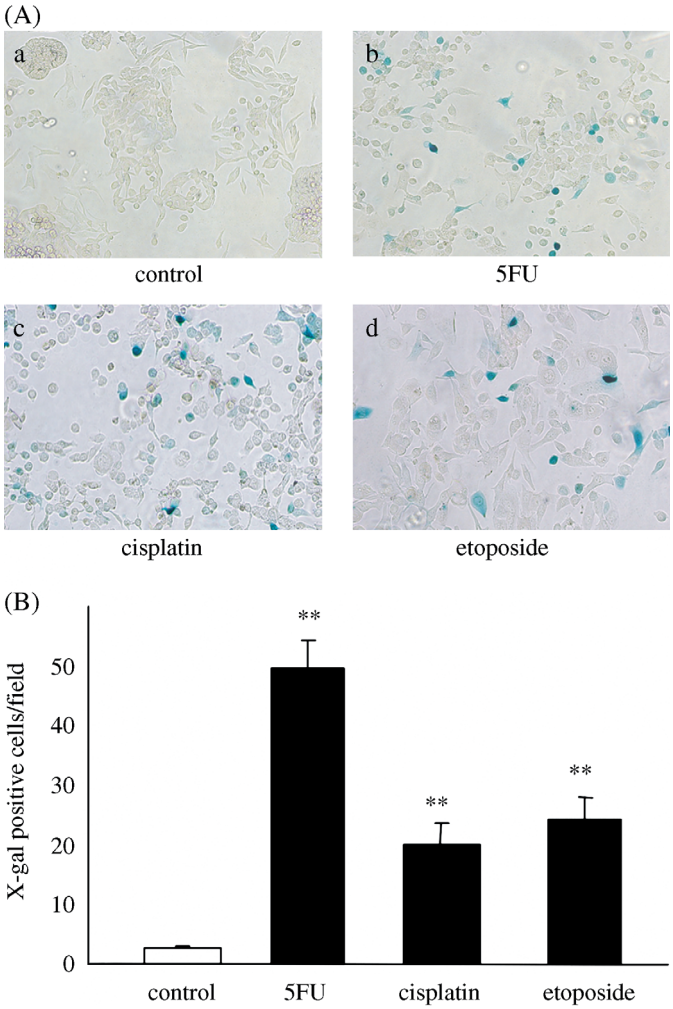

Fig. 2. 5-fluorouracil (5FU), cisplatin and etoposide significantly increase β-galactosidase (β-gal) expression by Ad-lacZ-infected cells. SUIT-2 cells were treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM) for 24 h and then infected with Ad-lacZ at a multiplicity of infection (MOI) of 10. β-gal activity was assessed by X-gal staining at 48 h after infection. (A) Photomicrographs of X-gal-stained control or treated cell cultures (×100). a, control; b, 5FU; c, cisplatin; d, etoposide. (B) Numbers of β-gal-positive control or treated cells per field. Each value represents the mean ± SD of five independent fields. $^{**}P < 0.01$, compared with control cells.

Effects of 5FU, cisplatin and etoposide on β-gal expression by Ad-lacZ-infected cells. To investigate the effects of the chemotherapeutic agents on the expression of another gene delivered by an adenoviral vector, we used Ad-lacZ instead of Ad-NK4 and examined the expression of β-galactosidase by the transfected cells. SUIT-2 cells (2×10^5) were treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM) for 24 h, and then infected with Ad-lacZ at an MOI of 10. At 48 h after infection, the cells were stained for β-gal. As shown in Fig. 2(A), large numbers of cells treated with 5FU, cisplatin or etoposide showed the characteristic blue staining indicative of β-gal activity, while only small numbers of untreated cells were positive for β-gal. The numbers of β-gal-positive cells in five independent fields were significantly higher for the treated cells than for the untreated cells (Fig. 2B, $P < 0.001$). These data are consistent with those of our Ad-NK4 experiments.

Effects of 5FU, cisplatin and etoposide on Ad-NK4-mediated inhibition of HGF-induced invasion of pancreatic cancer cells. We previously reported that NK4 inhibits HGF-induced invasion of pancreatic cancer cells.[30,31] In the present study, we tested the

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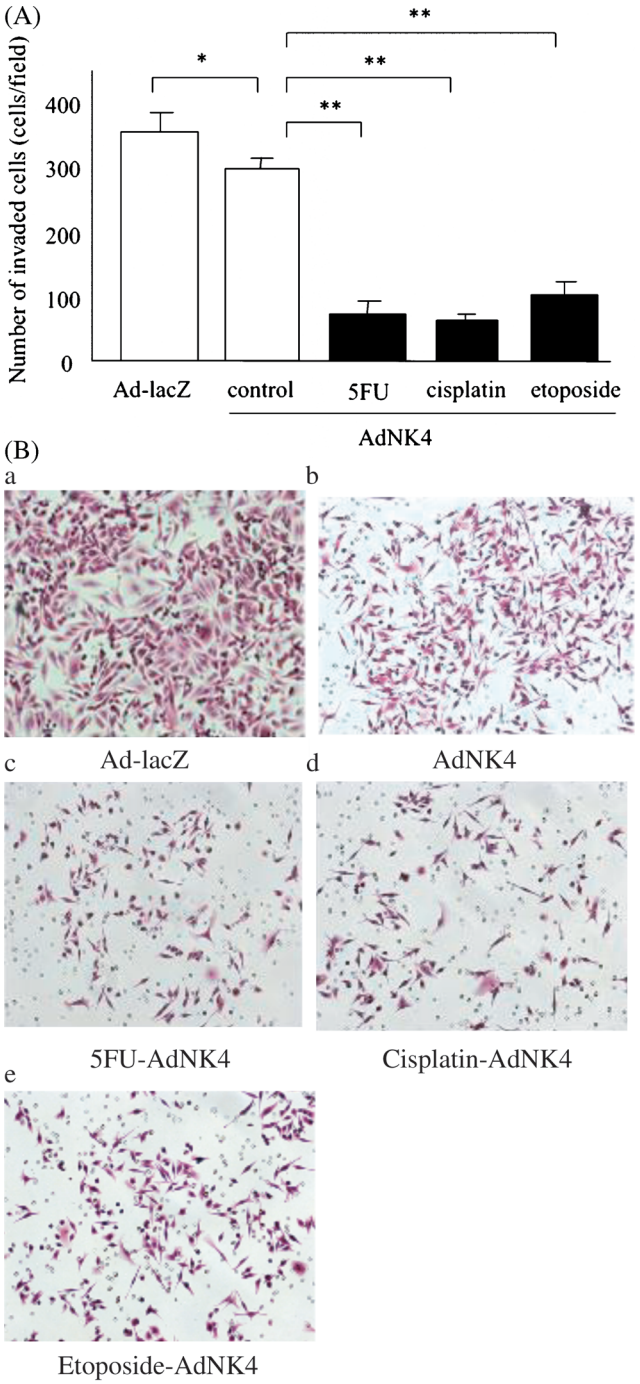

Fig. 3. 5-fluorouracil (5FU), cisplatin and etoposide significantly enhance Ad-NK4-mediated inhibition of hepatocyte growth factor (HGF)-induced invasion. SUIT-2 cells (1×10^7) were left untreated or treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM) for 24 h. After removing the media containing chemotherapeutic agents, the cells were then infected with Ad-lacZ or Ad-NK4 at a multiplicity of infection (MOI) of 50 in fresh media, and the conditioned media were collected on postinfection day 3. New SUIT-2 cells were seeded into the upper chambers of 24-well plates and then exposed to each of the five precollected conditioned media in the presence of 0.01 ng/mL HGF for 72 h. (A) Numbers of cells that invaded to the lower surface of the Matrigel-coated membranes. Each value represents the mean ± SD of five randomly selected fields. $^{**}P < 0.01$. $^{*}P < 0.05$. (B) Photomicrographs of *in vitro* invasion assays with SUIT-2 cells cultured with conditioned media derived from Ad-lacZ-infected cells (a), Ad-NK4-infected cells (b), Ad-NK4-infected cells pretreated with 5FU (c), Ad-NK4-infected cells pretreated with cisplatin (d) or Ad-NK4-infected cells pretreated with etoposide (e). Hematoxylin and eosin stain (×100).

biological effects of the 5FU-, cisplatin- and etoposide-induced increases in NK4 expression by Ad-NK4 on the inhibition of invasion of pancreatic cancer cells. SUIT-2 cells (1×10^7) were left untreated or treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM) for 24 h. After removing media containing chemotherapeutic agents, the cells were then infected with Ad-lacZ or Ad-NK4 at an MOI of 50 in fresh media, and conditioned media collected on postinfection day 3 were used for the following invasion assay. We used an *in vitro* invasion assay to examine the inhibitory effects of the five different conditioned media derived from Ad-lacZ-infected cells, Ad-NK4-infected cells, Ad-NK4-infected cells pretreated with 5FU, Ad-NK4-infected cells pretreated with cisplatin, or Ad-NK4-infected cells pretreated with etoposide, on the HGF (0.01 ng/mL)-induced invasiveness of new SUIT-2 cells. The number of invading cells cultured with conditioned media from SUIT-2 cells infected with Ad-NK4 was less than that of cells cultured with conditioned media from SUIT-2 cells infected with Ad-lacZ ($P = 0.011$), consistent with a previous report.[29] We further found that the conditioned media from SUIT-2 cells treated with each of the three chemotherapeutic agents prior to infection with Ad-NK4 significantly inhibited the invasiveness of pancreatic cancer cells compared with the conditioned media from untreated cells ($P < 0.001$) (Fig. 3A,B). To investigate the effects of Ad-NK4-induced NK4 expression enhanced by pretreatment of cells with 5FU-, cisplatin- and etoposide on the proliferation of pancreatic cancer cells, we collected conditioned media of SUIT-2 cells infected by Ad-NK4 with or without pretreatment with chemotherapeutic agents, and evaluated the proliferation of SUIT-2 cells cultured with these conditioned media 3 days after treatment. We found no differences among the groups examined (Fig. S1), consistent with previous reports.[46] These data suggest that not only was the adenovirus-delivered NK4 expression enhanced by the chemotherapeutic agents, but the increased NK4 also exerted biological effects as an antagonist of HGF.

Effects of 5FU, cisplatin and etoposide on adenovirus uptake. Next, we investigated the effects of 5FU, cisplatin and etoposide on adenovirus uptake by pancreatic cancer cells. SUIT-2 cells were treated with 5FU (2, 5 or 10 μM), cisplatin (1, 2 or 5 μM) or etoposide (2, 5 or 10 μM) for 24 h, and then infected with Ad-lacZ at an MOI of 10. At 24 h after infection, the viral DNA contents were quantified by real-time PCR. As shown in Fig. 4(A), the viral DNA contents of the agent-treated cells at 24 h were significantly higher than that of untreated cells ($P < 0.01$). These data suggest that 5FU, cisplatin and etoposide increase adenovirus uptake in dose-dependent manners.

Effects of post-treatment of cells with chemotherapeutic agents on Ad-NK4-induced NK4 expression. To investigate the effects of chemotherapeutic agents on adenovirus-mediated gene transfer independently of alteration of adenovirus infection at early phase, we infected cells with Ad-NK4 prior to treatments with 5FU, cisplatin, or etoposide. All three chemotherapeutic agents increased NK4 expression (Fig. 4B), which was similar to the results shown in Fig. 1, although the increases were relatively small.

Effects of post-treatment of cells with chemotherapeutic agents on expression driven by the CMV promoter. To evaluate the effect of chemotherapeutic agents on the CMV promoter used by Ad-NK4 and Ad-lacZ to drive expression of the target gene, SUIT-2 cells (5×10^6) were transfected with a plasmid expressing NK4 under the control of the CMV promoter, incubated for 24 h, and then left untreated or treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM). Culture media were collected on day 1 after treatment, and NK4 expression was measured. We observed no significant differences in NK4 expression among the groups examined (Fig. 4C). We also measured NK4 expression in culture media on days 2 and 3 after treatment, and found similar results (data not shown).

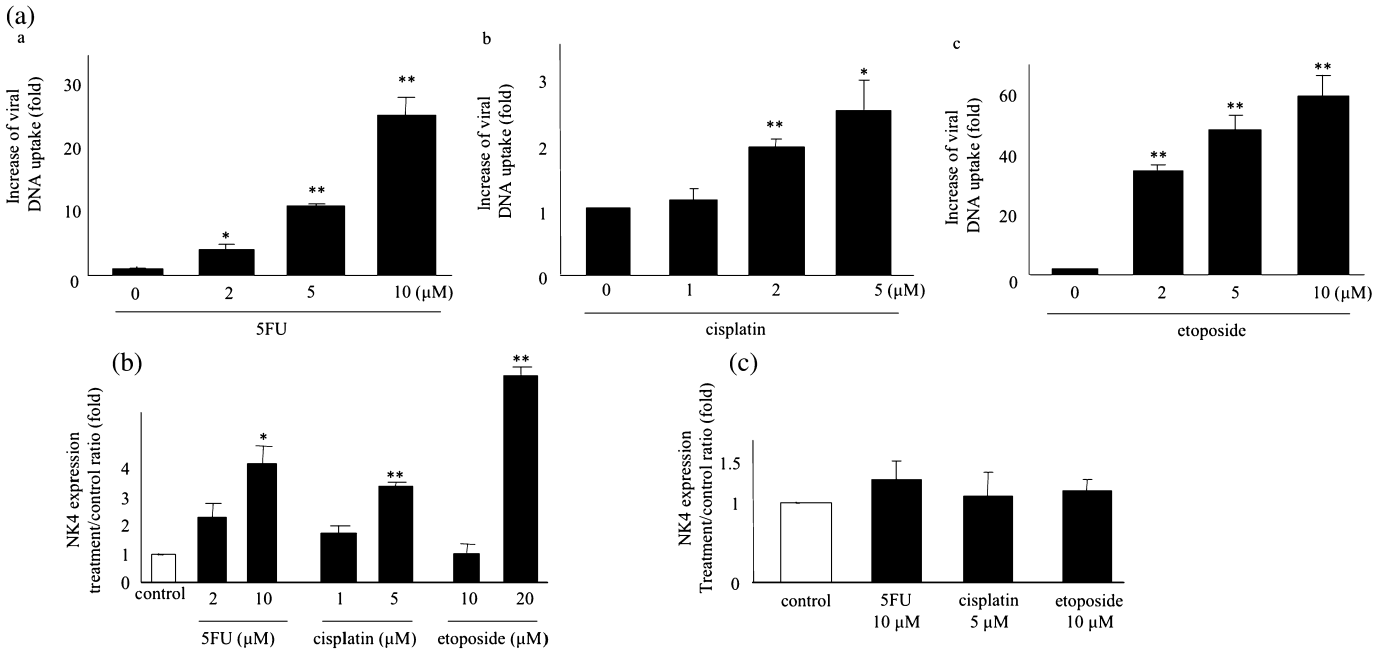

Fig. 4. 5-fluorouracil (5FU), cisplatin and etoposide increase adenovirus infection but not CMV promoter activity. (A) SUIT-2 cells were treated with 5FU (a), cisplatin (b) or etoposide (c) for 24 h and then infected with Ad-lacZ at a multiplicity of infection (MOI) of 10. DNA was extracted at 24 h after the infection. The viral DNA contents were quantified by real-time polymerase chain reaction and expressed as the fold increases relative to untreated cells. Each value represents the mean ± SD of triplicate measurements. $^{**}P < 0.01$. $^{*}P < 0.05$, compared with control cells. (B) Post-treatment of cells with 5FU, cisplatin and etoposide increases NK4 expression in Ad-NK4-infected cells. SUIT-2 cells were infected with Ad-NK4 at an MOI of 10 prior to treatment with 5FU, cisplatin or etoposide. NK4 levels in the culture media were measured by enzyme-linked immunosorbent assay on postinfection day 2. Each value represents the mean ± SD of three independent samples. $^{**}P < 0.01$. $^{*}P < 0.05$, compared with control cells. (C) SUIT-2 cells were transfected with NK4-expressing plasmids and then treated with 5FU (10 μM), cisplatin (5 μM) or etoposide (10 μM) at 24 h after transfection. NK4 concentrations in culture media were measured on post-treatment day 1. Each value represents the mean ± SD of three independent samples.

Effects of 5FU, cisplatin and etoposide on the expression levels of CAR, dynamin 2 and β3-integrin. For gene expression, adenoviruses require sequential steps of binding to a cell, endocytosis, endosomal escape, intracellular trafficking and nuclear delivery.[47] Endocytosis of adenoviruses mediated by clathrin-coated vesicles[48,49] requires the action of the large guanosine triphosphatase (GTPase) dynamin as a constrictase.[50] It was recently reported that radiation induces adenovirus infection via dynamin 2 in colon, brain, breast and pancreatic cancers[43,44,51] and that etoposide acts via CAR.[21] To investigate the effects of 5FU, cisplatin and etoposide on the expression levels of dynamin 2 and CAR in pancreatic cancer cells, we quantified the *dynamin 2* and *CAR* mRNA levels in SUIT-2 cells using real-time RT-PCR. We found that the *dynamin 2* mRNA expression levels were significantly higher in cells treated with 5FU (10 μM) and cisplatin (10 μM) at 4 h after treatment (5FU, $P = 0.033$; cisplatin, $P = 0.042$), while cells treated with etoposide were not affected (Fig. 5A). On the other hand, *CAR* mRNA expression was only affected in cells treated with 5FU ($P = 0.013$) (Fig. 5B). We also examined the expression levels of *clathrin* mRNA after treatment and found no changes after treatment with all three chemotherapeutic agents (data not shown).

Recently, other cell surface adenovirus receptors, namely α_v-integrin, β_3-integrin and β_5-integrin, have been reported to trigger cytoskeletal changes for endocytosis and mediate endosomal escape.[47] Therefore, we investigated the changes in the expression levels of these receptors. The results revealed that *β_3-integrin* mRNA expression was significantly enhanced by 5FU ($P = 0.0019$, Fig. 5C), while the expression levels of the other two receptors were not significantly affected (data not shown).

Effects of 5FU, cisplatin and etoposide on the expression of NK4 delivered by Ad-NK4 in mice xenografts. To evaluate the effects of the chemotherapeutic agents on NK4 expression in pancreatic cancers infected with Ad-NK4 *in vivo*, we established subcutaneous tumors treated with 5FU (10 mg/kg; $n = 6$), cisplatin (5 mg/kg; $n = 6$) or etoposide (5 mg/kg; $n = 6$) and untreated tumors ($n = 5$) in SCID mice. At 24 h after administration of the chemotherapeutic agents into the peritoneal cavity (i.p), 5×10^7 pfu of Ad-NK4 (100 μL) was injected into each subcutaneous tumor. To examine the levels of NK4 protein expression in the subcutaneous tumors, the mice were killed at 48 h after the administration of Ad-NK4, and the NK4 concentrations in the tumor lysates were measured by ELISA. The tumors treated with 5FU, cisplatin and etoposide expressed 5.4 ± 1.4-fold ($P = 0.006$), 11.8 ± 2.6-fold ($P < 0.001$) and 4.9 ± 1.8-fold ($P = 0.017$) higher levels of NK4 than the untreated tumors, respectively (Fig. 6). These results are consistent with our *in vitro* data and suggest that chemotherapeutic agents can enhance the expression of a target gene delivered by an adenovirus vector *in vivo*.

Discussion

In the present study, we found that the chemotherapeutic agents 5FU, cisplatin and etoposide could enhance the expression of target genes delivered to pancreatic cancer cells by adenovirus-based vectors in dose-dependent manners. Previously, many researchers have reported that adenoviruses enhance chemosensitivity.[22,52,53] Despite these previous reports focused on the effects of adenovirus-mediated gene therapy on chemotherapy, there are few reports regarding the effects of chemotherapy on adenovirus-mediated gene therapy. Recently, Reddy *et al.*[54] reported that cisplatin enhanced the apoptosis induced by an adenovirus expressing TNF related apotosis inducing ligand (TRAIL) in lung cancer, although the mechanism remained unclear. Furthermore, Shieh *et al.*[21] reported that low-dose etoposide enhanced adenoviral gene therapy through up-regulation of the human telomerase

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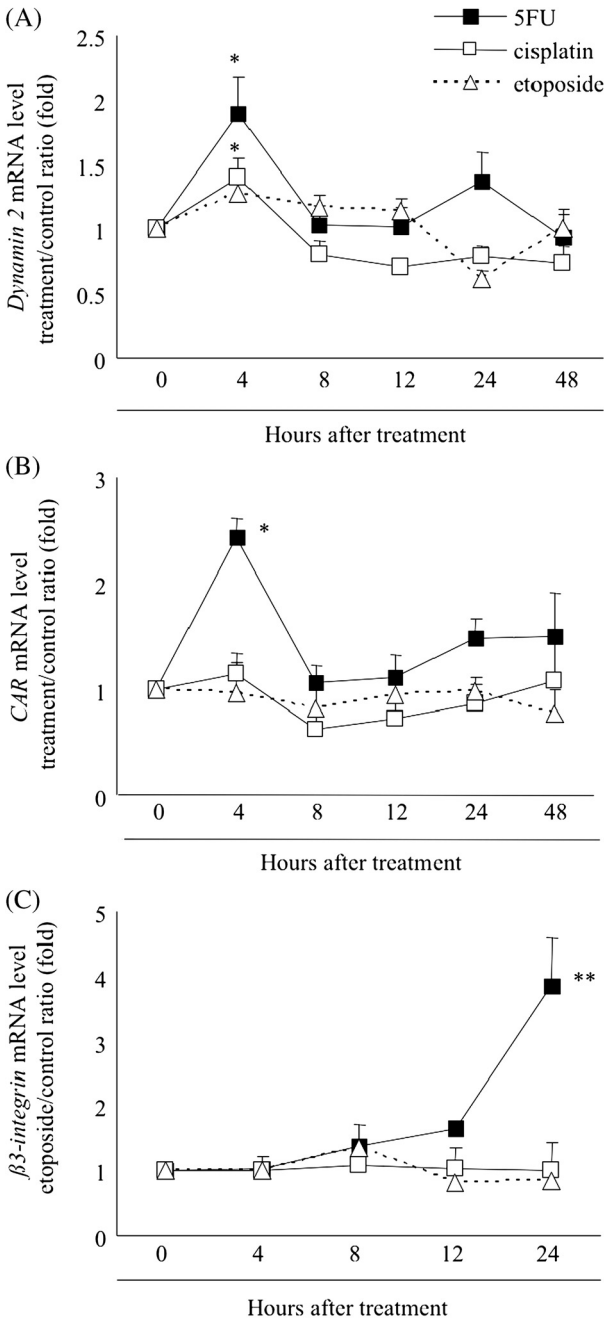

Fig. 5. Effects of 5-fluorouracil (5FU), cisplatin and etoposide on the expression levels of *dynamin 2*, *coxsackie virus and adenovirus receptor* (*CAR*) and *β3-integrin*. (A) *Dynamin 2* mRNA was quantified by real-time reverse transcription polymerase chain reaction from total RNA extracted from SUIT-2 cells treated with 5FU, cisplatin or etoposide at 4, 8, 12, 24 and 48 h after treatment and expressed as the fold increases relative to untreated cells. (B) *CAR* mRNA was quantified and expressed as described in (a). (C) *β3-integrin* mRNA was quantified and expressed as described in (A). Each value represents the mean ± SD of triplicate measurements. $^{**}P < 0.01$. $^{*}P < 0.05$, compared with 0 h cells.

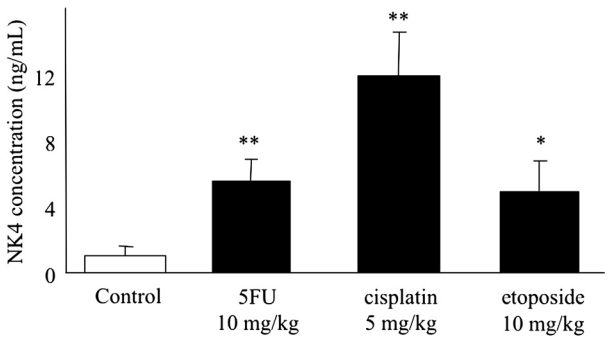

Fig. 6. 5-fluorouracil (5FU), cisplatin and etoposide significantly increase NK4 expression in subcutaneous tumors in mice. Subcutaneous tumors were established by injecting 5×10^6 SUIT-2 cells into back and both flanks of mice on day 0. At 7 days after the injection, six tumors were, respectively, treated with 5FU (10 mg/kg, i.p.), cisplatin (5 mg/kg, i.p.) or etoposide (10 mg/kg, i.p.), and five tumors were left untreated. At 24 h after intraperitoneal administration of chemotherapeutic agents, 5×10^7 pfu of Ad-NK4 (100 μL) was injected into each tumor. The mice were killed at 48 h after administration of Ad-NK4, and protein lysates of the tumors were adjusted to 10 mg/mL with lysis buffer. The NK4 concentrations in the tumor extracts were analyzed by enzyme-linked immunosorbent assay. The tumors treated with 5FU, cisplatin and etoposide express 5.4 ± 1.4-fold ($P = 0.006$), 11.8 ± 2.6-fold ($P < 0.001$) and 4.9 ± 1.8-fold ($P = 0.017$) higher levels of NK4 than the untreated tumors, respectively. Each value represents the mean ± SD of the NK4 expression in the six treated and five untreated tumors, respectively. $^{**}P < 0.01$. $^{*}P < 0.05$, compared with control tumors.

reverse transcriptase (hTERT) promoter activity and adenoviral infection. These recent data suggest that chemotherapy can be a new approach to enhance adenoviral gene therapy. Hecht *et al.*[55] reported a trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. Based on their results, endoscopic ultrasound injection of Ad-NK4 combined with intravenous chemotherapeutic agents may be one of the promising clinical protocols.

In the present study, we found that adenovirus-specific gene uptake in pancreatic cancer cells was increased after chemotherapy, suggesting that chemotherapeutic agents enhance adenovirus infection. Zhang *et al.*[43] reported that radiation improves viral gene uptake in human colon, breast and brain cancer cells, and indicated that the increased adenovirus uptake was mediated by up-regulation of dynamin 2. On the other hand, Shieh *et al.*[21] reported that increased adenovirus uptake was mediated by up-regulation of CAR in bladder cancer. Therefore, chemotherapeutic agents may affect different targets in the adenoviral infection pathway depending on the agent or the types of target cells. To investigate the effects of 5FU, cisplatin and etoposide on the expression levels of dynamin 2 and CAR in pancreatic cancer cells, we quantified the *dynamin 2* and *CAR* mRNA levels in SUIT-2 cells using real-time RT-PCR. 5FU and cisplatin significantly enhanced the expression levels of *dynamin 2* mRNA, while etoposide did not. Furthermore, 5FU significantly enhanced the expression of *CAR* mRNA, while the other two agents did not. 5FU alone enhanced the expression of β_3*-integrin* mRNA. Therefore, several other mechanisms may underlie chemotherapeutic agent-induced adenovirus uptake.

In the present study, we found that chemotherapeutic agents enhanced adenovirus-mediated gene transfer without alteration of adenovirus infection levels in the early phase. However, the effect of post-treatment of cells with chemotherapeutic agents was relatively small compared with those of pretreatments. The data suggest that there are several mechanisms for chemotherapeutic agent-induced enhancement of adenoviral gene transfer other than enhanced adenovirus infection in the early phase. Furthermore, we found no significant differences in activation of the CMV promoter that followed by a NK4 cDNA[40] between cells treated with chemotherapeutic agents and untreated cells. We also found that expression of the β_3-integrin receptor, which mediates endocytosis or endosomal escape of adenoviruses[47] was increased after treatment with 5FU. These data suggest that chemotherapy-induced adenovirus gene transfer can be mediated by altered efficiency of active penetration of the endosomal membrane and escape to the cytosol.

The mortality rate of pancreatic cancer remains the highest among cancers.[9] Although gene therapy with adenovirus vectors is a promising strategy for treatment of cancers, the antitumor effects of a single dose of adenovirus-mediated gene therapy is often insufficient in clinics[5,18,19] possibly due to limited transduction efficiency of the adenovirus vectors. In the present study, we found that chemotherapeutic agents, the dosages of which were similar to those used in the clinical setting[56–58] dramatically enhanced adenovirus-mediated gene expression, but were unable to clarify the mechanism for these effects. Further investigations of the underlying mechanism may lead to the discovery of new approaches that exert the same effects on adenoviral gene therapy as these agents. In conclusion, the present data suggest that chemotherapeutic agents significantly improve the efficiency of adenovirus-mediated gene transfer in pancreatic tumors. Furthermore, they will probably contribute to decreases in the adenovirus doses required for gene transfer, thereby controlling the side-effects of adenovirus infection in surrounding normal tissues.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Cell proliferation assays with new SUIT-2 cells cultured with precollected conditioned media derived from Ad-NK4-infected cells without chemotherapeutic agents (control), Ad-NK4-infected cells pretreated with 5-fluorouracil (5FU) (10 μM), Ad-NK4-infected cells pretreated with cisplatin (5 μM) or Ad-NK4-infected cells pretreated with etoposide (5 μM). Each value represents the mean ± SD of proliferation ratio of triplicate wells to control at postseeding day 3.