Radiation Enhances Adenoviral Gene Therapy in Pancreatic Cancer via Activation of Cytomegalovirus Promoter and Increased Adenovirus Uptake

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Abstract

Purpose: Adenovirus-mediated gene therapy combined with radiation is expected to be a new approach to treat pancreatic cancer. However, there are no reports of definitive effects of radiation on adenovirus-mediated gene therapies. In the present study, we investigated the effect of radiation on the transduction efficiency of an adenovirus-based gene therapy.

Experimental Design: We used adenovirus expressing NK4 (Ad-NK4), an antagonist for hepatocyte growth factor, as a representative gene therapy. Pancreatic cancer cells preinfected with Ad-NK4 were irradiated, and NK4 levels in culture media of these cells were measured. We investigated cytomegalovirus (CMV) promoter activity and uptake of adenovirus in these cells. To examine the effect of radiation in vivo, Ad-NK4 was given to irradiated subcutaneous tumors in nude mice, and NK4 levels in tumors were measured.

Results: NK4 levels in culture media of irradiated cells were 4.5-fold (P < 0.01) higher than those of nonirradiated cells. Radiation enhanced activation of the CMV promoter and adenovirus uptake (P < 0.01), leading to increased levels of NK4. We found that activation of p38 mitogen-activated protein kinase and up-regulation of dynamin 2 may be involved in the radiation-induced activation of the CMV promoter and adenovirus uptake, respectively. NK4 levels in irradiated tumors were 5.8-fold (P = 0.017) higher than those in nonirradiated tumors.

Conclusions: The present findings suggest that radiation significantly improves the efficiency of adenovirus-mediated gene transfer in pancreatic cancer and probably contributes to decreasing the dose of adenovirus required for gene transfer and controlling side effects of adenovirus infection in nonirradiated normal tissue.

On the basis of recent advances in our understanding of the molecular biology of a variety of cancers (1–4), molecular therapies, which target tumor-specific pathways and interfere with key regulatory cellular functions, including proliferation, differentiation, metastasis, and survival of cancer cells, have been extensively studied (5, 6). 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 reaction, such as skin rash (5). The use of viral vectors, which have high gene transfer efficiencies, is one approach for molecular therapy and is often useful for expressing an antagonist of a target protein. Adenovirus-based vectors are often used due to their high transduction efficiency and high levels of transient expression of the transfected gene (7).

Pancreatic cancer is a leading cause of cancer-related death in industrial countries (8, 9). Most patients with pancreatic cancer have poor outcomes because early diagnosis is difficult and because conventional therapies have limited effectiveness (10). Recently, advances in our understanding of the genetics and epigenetics of pancreatic cancer revealed that alterations of several tumor-related genes, including K-ras, p53, matrix metalloproteinase, hepatocyte growth factor (HGF), and epidermal growth factor receptor (11–16), may underlie the aggressiveness of this neoplasm and its resistance to conventional therapies (6). Therefore, molecular therapies for pancreatic cancer are promising new approaches to treat this often fatal disease. Investigators have used adenovirus-mediated gene transfer to treat pancreatic cancer and reported that adenovirus-mediated gene therapy inhibited progression of pancreatic cancer in vivo and in vitro (17, 18). However, clinical trials revealed that it is...
difficult to eradicate pancreatic tumors with adenovirus-mediated gene therapy alone (19, 20). There is also concern that the dose of adenovirus necessary to achieve therapeutic effectiveness may have some significant toxicity. Therefore, adenovirus alone may not be an effective treatment for cancer, and it may be necessary to combine adenovirus-mediated gene therapies with conventional treatments to maximize the antitumor effects for pancreatic cancer.

Recently, the combination of radiotherapy and adenovirus-mediated gene therapy has been reported to be effective for cancer treatment. Shi et al. (21) reported that adenovirus-mediated gene therapy targeting endostatin enhanced the antitumor effect of radiation therapy in colorectal cancer. Similarly, Geoerger et al. (22), Portella et al. (23), and Rogulski et al. (24) reported that ONYX-015, an E1B–55-kDa gene-deleted adenovirus that replicates selectively in and lyses tumor cells with abnormalities in p53 function, combined with radiation therapy is a promising strategy for treatment of gliomas and thyroid cancers and that there are synergistic effects with such combination therapies. Previously, we reported that gene therapy with an adenovirus vector expressing NK4 (Ad-NK4), which acts as an HGF antagonist, could be a viable option for treatment of pancreatic cancer (25–27). More recently, we reported that radiation therapy enhances the invasiveness of pancreatic cancer cells via the activation of the HGF receptor c-Met and that NK4 inhibits this radiation-enhanced invasiveness (28, 29), suggesting that a combination of radiation therapy and NK4 gene therapy may be a viable strategy for treatment of pancreatic cancer. However, the effects of radiation on features of adenovirus-mediated gene therapies, such as adenovirus uptake and efficiency of target gene expression, have remained unknown.

In the present study, to investigate the effect of radiation on the efficiency of transfer and expression of a target gene, we examined the effect of radiation on NK4 expression by an adenovirus-based vector (Ad-NK4) as a representative gene therapy. We found that radiation increased expression of NK4 via enhanced activation of the cytomegalovirus (CMV) promoter, which is commonly used as the target gene promoter in adenovirus vectors, and we also found that radiation enhanced

![Fig. 1.](image-url)
uptake of the adenovirus vector. The present data also suggest that activation of p38 mitogen-activated protein kinase (MAPK) and up-regulation of dynamin 2 may be involved in the radiation-enhanced activation of CMV promoter and adenovirus uptake, respectively.

Materials and Methods

Cells and reagents. Human pancreatic cancer cell lines SUIT-2, AsPC-1, PANC-1, and KP-1N were generously donated by Dr. H. Iguchi (National Shikoku Cancer Center) and cultured in DMEM supplemented with streptomycin, penicillin, and 10% fetal bovine serum at 37°C in 5% CO₂. The p38 inhibitor SB203580 was purchased from Calbiochem. Human recombinant HGF was purified from the conditioned medium of Chinese hamster ovary cells expressing human HGF cDNA (30, 31).

Construction of recombinant adenovirus. A recombinant Ad-NK4 was constructed as described previously (32). In brief, Ad-NK4 was generated by homologous recombination of the pJM17 plasmid (33) and the shuttle plasmid vector pSV2+ (34) containing an expression cassette and the CMV early promoter/enhancer followed by human NK4 cDNA (35) and a polyadenylation signal. A control vector expressing the bacterial β-galactosidase gene (lacZ) was constructed by the same procedure with pJM17 and pCA17, which contains the lacZ gene. Recombinant Ad-NK4 and Ad-lacZ were propagated in HEK293 cells.

Radiation treatment. Cells were irradiated with a dose of 2, 5, or 10 Gy at room temperature with a 137Cs source (Gamma Cell 40; Atomic Energy of Canada, Ltd.) with a delivery rate of 1.0 Gy/min.

Adenovirus infection of cells. Cells (5 × 10⁴) were seeded in six-well plates and cultured in DMEM supplemented with 10% fetal bovine serum for 24 h. Cells were treated with or without radiation and then infected with Ad-NK4 or Ad-lacZ at multiplicities of infection (MOI) of 10 or MOI of 50 at 24 h after radiation treatment. The culture medium was replaced with fresh medium 1.5 h after transfection.

Extraction of proteins from cells infected with Ad-NK4. SUIT-2 cells were irradiated and infected with Ad-NK4, as described above. Two days after infection with Ad-NK4, the cells were lysed in 500 μl ice-cold lysis buffer [150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L EDTA, 5 μg/ml leupeptin, 1 mmol/L phenylmethyl sulfonyl fluoride, and 0.5% (v/v) Triton X-100]. Cell debris was removed by centrifugation at 14,000 × g for 20 min at 4°C, supernatants were collected, and the protein concentrations were measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) at absorbances of 280 nm and adjusted to 2.0 mg/mL with lysis buffer.

Electroporation. pcDNA3-NK4 (2.5 μg; NK4-expressing plasmid) or pcDNA3 (2.5 μg; empty vector) was mixed with 5 × 10⁶ SUIT-2 cells and electroporated with a Nucleofector (Amaxa Biosystems GmbH) according to the manufacturer’s instructions. The cells were immediately transferred to complete medium prewarmed to 37°C and allowed to recover for 24 h.

NK4 expression by Ad-NK4–infected or NK4-expressing plasmid–transfected cancer cells. After infection of SUIT-2, AsPC-1, PANC-1, and KP-1N cells with Ad-NK4 or transfection of SUIT-2 with NK4-expressing plasmid, the medium was changed every 24 h. Conditioned media were collected on posttransduction days 1, 2, 3, and 4. The NK4 concentration in the media was measured by ELISA with a human HGF ELISA kit (Immunis HGF EIA, Institute of Immunology) according to the manufacturer’s instructions. The cells were immediately transferred to complete medium prewarmed to 37°C and allowed to recover for 24 h.

Invasion assay. Invasiveness of pancreatic cancer cells was quantified as the number of cells invading through Matrigel-coated transwell inserts (Becton Dickinson) as described previously (36). In brief, transwell inserts with 8-μm pores were coated with Matrigel (20 μg/well;
Becton Dickinson). SUIT-2 cells (1 × 10^3) were untreated or irradiated with 10 Gy and then allowed to recover for 24 h. These cells were infected with Ad-lacZ or Ad-NK4 at MOI of 50, and culture media were collected on postinfection day 3. New untreated SUIT-2 cells were seeded in 24-well plates at a density of 1 × 10^3/cm^2 in the upper chamber in 250 μL of DMEM supplemented with 10% fetal bovine serum and cultured with 750 μL of conditioned media from the irradiated or untreated SUIT-2 cells infected with Ad-lacZ or Ad-NK4. After 24 h of incubation in the presence of 3 ng/mL HGF, cells that had invaded to the lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with H&E, and counted in five randomly selected fields under a light microscope.

*Western blotting of phosphorylated p38 MAPK.* Untreated or irradiated SUIT-2 cells were lysed in ice-cold lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/LDTT, 1 mmol/L orthovanadate, 1 mmol/L phenylmethyl sulfonlfuoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin (pH 7.4)]. The lysates were boiled for 10 min, and the proteins were fractionated by 8% SDS-PAGE and transferred to a polyvinylidine difluoride membrane (Millipore). The membrane was incubated overnight at 4°C with rabbit polyclonal antiphosphorylated p38 MAPK antibody (1:1,000; Cell Signaling Technology) and then probed with antirabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology).

*Assessment of transgene distribution by evaluation of β-galactosidase expression.* At 48 h after adenovirus infection, SUIT-2 cells were rinsed twice with PBS and fixed with 0.25% glutaraldehyde in PBS for 15 min at 4°C. β-Galactosidase activity was detected by immersing cells into solution (5 mmol/L K4FeCN, 5 mmol/L K3FeCN, and 2 mmol/L MgCl2 containing 1 mg/mL X-gal) at 4°C for 6 h.

*Real-time PCR and reverse transcription–PCR assays.* Ad-lacZ DNA content of infected cells was determined by real-time PCR analysis as described previously (37) with primers for the β-galactosidase gene (5′-CACGCCGAGATAGATGGCT-3′ and 3′-ATCCCGATTGACCCAT-CC-5′; ref. 38). The number of copies of viral DNA was calculated from a standard curve of purified adenovirus vector (CMV–β-galactosidase) and was further adjusted to the protein concentration of each lysate. *Dynamin 2* mRNA levels were quantified with 100 ng of total RNA by real-time reverse transcription–PCR assay with a QuantiTect SYBR Green reverse transcription–PCR kit (Qiagen) and primers specific for *dynamin 2* (5′-AGGATCTGGTGTGCAGTGC-3′ and 3′-GTCATGTGGTCATGTCAGG-5′; ref. 38). Levels of *dynamin 2* mRNA were normalized to those of 18S rRNA amplified with specific primers (5′-GTCACCCGTTGAACCCCAAT and 3′-GCCAGTGATGGAACCTACC; ref. 39) and expressed as a ratio compared with nonirradiated controls.

*Inhibition of dynamin 2 in cells by RNA interference.* SUIT-2 cells were transfected with *dynamin 2*–specific short interfering RNA (siRNA; Dharmacon) or control siRNA provided by Qiagen with a Nucleofector (Amaxa) and plated at 1 × 10^5 cells per well in six-well plates. At 24 h after transfection, cells were irradiated or left untreated. At 48 h after transfection, the cells were infected with Ad-lacZ at MOI of 10, as described above. Viral gene uptake in *dynamin 2*–specific siRNA-transfected cells is expressed as a ratio compared with that in control siRNA-transfected cells.

*Evaluation of radiation-induced expression of NK4 in vivo in xenografts in nude mice.* Six-week-old female nude mice (BALB/c nu/nu) were obtained from Japan SLC. To investigate the radiation-induced therapeutic effect of Ad-NK4 in vivo, 10 subcutaneous tumors were preestablished in five nude mice by injection of 5 × 10^6 SUIT-2 cells into both flanks. Seven days later, six tumors in three mice were irradiated with 10 Gy and four tumors in two mice were untreated, and at 24 h after radiation, 5 × 10^6 plaque-forming units of Ad-NK4 (100 μL) were injected into the tumors with a 26-gauge needle. Tumors were irradiated locally with animals restrained in a custom lead block. To examine expression of NK4 protein in subcutaneous tumors, mice were killed 48 h after administration of Ad-NK4 and tumors were excised. The samples were immediately washed once in PBS and homogenized with (300 μL/tumor) protein lysis buffer [50 mmol/L NaCl, 30 mmol/L sodium PPI, 50 mmol/L NaF, 5 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin (pH 7.4), 0.25% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin (pH 7.4)]. The lysates were boiled for 10 min, and the proteins were fractionated by 8% SDS-PAGE and transferred to a polyvinylidine difluoride membrane (Millipore). The membrane was incubated overnight at 4°C with rabbit polyclonal antiphosphorylated p38 MAPK antibody (1:1,000; Cell Signaling Technology) and then probed with antirabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology).

Fig. 3. Radiation significantly enhances Ad-NK4–mediated inhibition of HGF-induced invasion. SUIT-2 cells (1 × 10^3) were nonirradiated or irradiated with 10 Gy and then allowed to recover for 24 h. Cells were then infected with Ad-lacZ or Ad-NK4 at MOI of 50, and culture media were collected on postinfection day 3. Untreated new SUIT-2 cells were seeded in the upper chambers of 24-well plates and then exposed to one of the four different conditioned media in the presence of 3 ng/mL HGF for 24 h. A, number of cells that invaded to the lower surface of the Matrigel-coated membrane. Columns, mean of five randomly selected fields; bars, SD. **P < 0.01. B, a. photomicrographs of *in vitro* invasion assay with SUIT-2 cells cultured with conditioned media from SUIT-2 cells infected with Ad-NK4 at MOI of 50 without radiation. b, photomicrographs of SUIT-2 cells cultured with conditioned media from SUIT-2 cells infected with Ad-NK4 after radiation treatment (10 Gy). H&E stain. Magnification, 100×.
and 0.1% bovine albumin], and protein concentrations were measured as described above and adjusted to 10.0 mg/mL with lysis buffer. The NK4 concentration in the extract was analyzed by ELISA (Immunis HGF EIA).

Statistical analysis. Values are expressed as mean ± SD. Comparisons between all groups were analyzed by one-way ANOVA and Student’s t test for comparison between the two groups. The level of statistical significance was set at P < 0.01 or P < 0.05. To confirm the induction results, experiments were repeated at least thrice.

Results

Effect of radiation on expression of target genes delivered by adenoviral vector. To investigate the effect of radiation on the expression of a target gene delivered with an adenoviral vector, we measured expression of NK4 in culture media of pancreatic cancer cells infected with Ad-NK4 with or without radiation. SUIT-2 cells were transfected with NK4-expressing plasmid and then irradiated with 10 Gy at 24 h after transfection. NK4 concentrations in culture media were measured on postirradiation days 0, 1, and 3. Points, mean of three independent samples; bars, SD. *, P < 0.05. B, response of phosphorylated p38 to radiation (10 Gy). SUIT-2 cells were nonirradiated or irradiated with 10 Gy, and proteins were isolated at 12 and 24 h after radiation and subjected to Western blot analysis with antibodies that specifically recognize activated phosphorylated p38 or the indicated proteins. C, effect of p38 MAPK inhibitor SB203580 on NK4 expression by NK4-expressing plasmid – transfected cells with or without radiation. SUIT-2 cells transfected with NK4-expression plasmid were incubated in the presence or absence of p38 inhibitor (10 μmol/L) and irradiated with 10 Gy or nonirradiated at 24 h after transfection. NK4 concentrations in culture media were measured on postirradiation day 1. The culture medium and p38 inhibitor were replaced every 24 h. Columns, mean of three independent samples; bars, SD. **, P < 0.01; *, P < 0.05. D, SUIT-2 cells transfected with NK4-expressing plasmid were incubated in the presence or absence of p38 inhibitor SB203580 (0, 1, 5, or 10 μmol/L) and irradiated with 10 Gy. NK4 concentrations in culture media were measured on postirradiation day 1. The medium and p38 inhibitor were replaced every 24 h.

Extracted proteins from irradiated SUIT-2 cells infected with Ad-NK4, as described above, and measured levels of NK4. As shown in Fig. 1B, radiation also significantly increased intracellular NK4 protein levels in a dose-dependent manner. These data suggest that radiation enhances expression of a target gene delivered by adenovirus vector.

Effect of radiation on β-galactosidase expression by Ad-lacZ–infected cells. To investigate the effect of radiation on the expression of another gene delivered with an adenoviral vector, we used Ad-lacZ instead of Ad-NK4 and examined expression of β-galactosidase by transfected cells. SUIT-2 cells (2 × 10^5) were irradiated with 10 Gy, allowed to recover for 24 hours, and then infected with Ad-lacZ at MOI of 10. At 48 hours after infection, cells were stained for β-galactosidase. As shown in Fig. 2A, a large number of irradiated cells showed the characteristic blue staining indicative of β-galactosidase activity, but only a small number of nonirradiated cells were positive for β-galactosidase. The numbers of β-galactosidase–positive cells in five independent fields were counted, and the percentage of β-galactosidase–positive irradiated cells was significantly larger than that of nonirradiated cells (P < 0.01; Fig. 2B). These data are consistent with those of our Ad-NK4 experiments.

Effect of radiation 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 (25, 26). In the present study, we tested the effect of radiation on Ad-NK4–induced inhibition of invasion of pancreatic cancer cells. SUIT-2 cells (1 × 10^5) were treated with radiation and 0.1% bovine albumin, and protein concentrations were measured as described above and adjusted to 10.0 mg/mL with lysis buffer. The NK4 concentration in the extract was analyzed by ELISA (Immunis HGF EIA).

Statistical analysis. Values are expressed as mean ± SD. Comparisons between all groups were analyzed by one-way ANOVA and Student’s t test for comparison between the two groups. The level of statistical significance was set at P < 0.01 or P < 0.05. To confirm the induction results, experiments were repeated at least thrice.

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Statistical analysis. Values are expressed as mean ± SD. Comparisons between all groups were analyzed by one-way ANOVA and Student’s t test for comparison between the two groups. The level of statistical significance was set at P < 0.01 or P < 0.05. To confirm the induction results, experiments were repeated at least thrice.
or without 10 Gy and infected with Ad-lacZ or Ad-NK4 at MOI of 50 at 24 hours after radiation. The culture media were collected on postinfection day 3. We used an in vitro invasion assay to examine inhibition of HGF (3 ng/mL)-induced invasiveness of nonirradiated pancreatic cancer cells cultured with each of the four different conditioned media described above (0 Gy with Ad-lacZ, 10 Gy with Ad-lacZ, 0 Gy with Ad-NK4, or 10 Gy with Ad-NK4). The number of invading cells cultured in conditioned media from SUIT-2 cells infected with Ad-NK4 was less than that of cells infected with Ad-lacZ (P < 0.01). These data are consistent with those of previous reports (26). We also found that conditioned medium of SUIT-2 cells irradiated with 10 Gy before infection with Ad-NK4 significantly inhibited invasiveness of pancreatic cancer cells compared with that of nonirradiated cells (P < 0.01; Fig. 3A and B). These data suggest that radiation-enhanced adenovirus gene transfer improved efficiency of NK4-induced inhibition of invasion for treatment of pancreatic cancer.

**Effect of radiation on NK4 expression by pancreatic cancer cells transfected with NK4-expressing plasmid.** Ad-NK4 and Ad-lacZ use the CMV promoter to drive expression of the target gene. To evaluate the effect of radiation on the CMV promoter, we transfected SUIT-2 cells with a plasmid that expresses NK4 under the control of the CMV promoter before radiation treatment and measured NK4 expression after radiation. SUIT-2 cells (5 × 10⁶) were transfected with NK4-expressing plasmid or empty vector. Cells were incubated for 24 hours and then irradiated with 10 Gy. Culture media were collected on postradiation days 0, 1, and 3. NK4 expression in NK4-expressing vector-transfected cells is shown in Fig. 4A. Irradiated cells expressed significantly higher levels of NK4 than did nonirradiated cells (P < 0.05). NK4 expression was not detected in cells transfected with empty vector (data not shown). These data suggest that radiation increases the activity of the CMV promoter.

We next tested the effect of radiation on p38 MAPK and interaction between p38 MAPK and the CMV promoter. It has been reported that activation of p38 MAPK increases expression of transgenes driven by the CMV promoter (40, 41). Therefore, we tested whether radiation activates p38 MAPK and found that radiation increased expression of phosphorylated p38 MAPK (Fig. 4B). We also investigated the role of p38 MAPK in activation of the CMV promoter using the p38 inhibitor SB203580. SUIT-2 cells transfected with NK4-expressing vector were treated with or without SB203580 (10 μmol/L). Cells were then irradiated with 10 Gy, and the medium was replaced 24 hours after transfection. Culture media were collected on postradiation day 1. As shown in Fig. 4C, SB203580 reduced NK4 expression significantly in both irradiated cells and nonirradiated cells (irradiated cells, P < 0.01; nonirradiated cells, P < 0.05). NK4 expression in SUIT-2 cells treated with p38 inhibitor (0, 1, 5, or 10 μmol/L SB203580) on postradiation day 1 is shown in Fig. 4D. p38 inhibitor reduced radiation-enhanced NK4 expression in a dose-dependent manner. These data suggest that phosphorylation of p38 MAPK is involved in radiation-induced activation of the CMV promoter.

It is also possible that radiation activates the CMV promoter via activation of nuclear factor-κB. It has been reported that radiation activates nuclear factor-κB (42) and that the nuclear factor-κB pathway increases CMV promoter activity (43). Therefore, we examined the effect of radiation on activation of nuclear factor-κB in SUIT-2 cells and found that activation of nuclear factor-κB was not affected by radiation in the pancreatic cancer cell lines examined here (data not shown).

**Effect of radiation on adenoviral gene uptake.** We next investigated the effect of radiation on adenoviral gene uptake by pancreatic cancer cells. SUIT-2 cells were irradiated with 2, 5, or 10 Gy, allowed to recover for 24 hours, and then infected with Ad-lacZ at MOI of 10. At 24 hours after infection, the viral DNA content was quantified by real-time PCR. As shown in Fig. 5, the viral DNA content of cells at 24 hours after radiation was significantly higher than that of nonirradiated cells (P < 0.01). These data suggest that radiation increases viral gene uptake in a dose-dependent manner.

**Effect of radiation on viral infection and expression of dynamin 2.** Endocytosis of adenovirus mediated by clathrin-coated vesicles (44, 45) requires the action of the large GTPase dynamin as a constrictor (46). It was recently reported that radiation induces adenovirus infection via dynamin 2 in colon cancer, brain cancer, and breast cancer (37, 38). To investigate the effect of radiation on expression of dynamin 2 by pancreatic cancer cells, we quantified dynamin 2 mRNA levels in SUIT-2 cells by real-time reverse transcription–PCR. We found that dynamin 2 mRNA expression was significantly higher in irradiated cells (P < 0.05 at 12 hours after radiation) than in nonirradiated cells (Fig. 6A). We next used siRNA to inhibit dynamin 2 expression to determine whether dynamin 2 affects radiation-induced viral infection. We transfected SUIT-2 cells with a dynamin 2–specific siRNA or control siRNA and confirmed that dynamin 2 expression was significantly lower in cells transfected with the specific siRNA than in those transfected with control siRNA (Fig. 6B). Radiation enhanced Ad-lacZ uptake in the control siRNA-treated cells as much as 6.3 ± 0.8-fold, whereas Ad-lacZ uptake was significantly impaired in cells transfected with siRNA targeting dynamin 2 (P < 0.05; Fig. 6C). These data suggest that increased expression of dynamin 2 is involved in radiation-induced increase of adenovirus uptake. We also examined expression of clathrin mRNA after radiation and found that clathrin did not respond to radiation (data not shown). Although cell surface adenovirus receptors, such as coxsackie and adenovirus receptor and...
αv integrin receptor, may affect the efficiency of adenovirus infection, expression of the mRNAs for these receptors was not changed by irradiation (data not shown).

Effect of radiation on expression of NK4 delivered by Ad-NK4 in nude mice xenografts. To evaluate the effect of radiation on NK4 expression of pancreatic cancers treated with Ad-NK4 in vivo, we established six irradiated tumors and four untreated tumors. At 24 hours after radiation, $5 \times 10^7$ plaque-forming units of Ad-NK4 (100 μL) were injected into each tumor. To examine the expression of NK4 protein in the subcutaneous tumors, mice were killed 48 hours after administration of Ad-NK4, and the NK4 concentrations in the tumor lysates were measured by ELISA. Irradiated tumors expressed $5.8 \pm 3.5$–fold ($P = 0.017$) higher levels of NK4 than did nonirradiated tumors (Fig. 7). This result is consistent with our in vitro data and suggests that radiation can enhance expression of a target gene delivered by adenovirus vector in vivo.

Discussion

In the present study, we found that radiation enhances expression of target genes delivered to pancreatic cancer cells by an adenovirus-based vector in a dose-dependent manner. Furthermore, we investigated the mechanisms that underlie the radiation-enhanced target gene transfer and found that radiation increased activation of the CMV promoter through phosphorylation of p38 MAPK and increased adenoviral uptake through increased expression of dynamin 2.

Despite previous reports describing the combination of radiation therapy and adenovirus-mediated gene therapy, the mechanism by which radiation enhances the expression of adenovirus-mediated gene has remained unknown. In the present study, we found that radiation enhances expression of target gene via activation of the CMV promoter, which is commonly used to drive expression of target genes by adenovirus vectors. Breuning et al. (40) and Chen et al. (41) reported that activation of p38 MAPK increases expression of transgenes under control of the CMV promoter. We previously showed that radiation increases p42 and p44 MAPK activity under specific conditions (47). Our present data indicate that radiation also increases phosphorylation of p38 MAPK leading to
activation of the CMV promoter, suggesting that radiation-induced phosphorylation of p38 MAPK is involved in radiation-enhanced expression of target genes.

In the present study, we also found that adenovirus-specific gene uptake by pancreatic cancer cells increases after radiation, suggesting that radiation enhances infection by adenovirus. Zhang et al. (37) reported that radiation improves gene transfer efficiency in human colon cancer, breast cancer, and brain cancer cells. These data suggest that radiation may improve the efficiency of gene therapy not only for pancreatic cancer but also many cancers. It has been reported that dynamin 2 and clathrin are involved in virus infection (48, 49). We found that *dynamin* 2 mRNA levels increase after radiation, whereas clathrin expression is not affected by radiation. Although cell surface adenovirus receptors, such as coxsackie and adenovirus receptor and αv integrin receptor, are key factors in adenovirus infection, we have found that expression of mRNAs for these molecules is not altered by radiation (data not shown). Qian et al. (38) reported that dynamin 2 mediates radiation-induced adenovirus infection in colon, brain, and breast cancer cell lines. In the present study, inhibition of dynamin 2 production by RNA interference significantly reduced Ad-lacZ uptake. These data suggest that dynamin 2 might be involved in radiation-induced adenovirus infection in pancreatic cancer cells.

The mortality rate of pancreatic cancer remains highest among cancers (10). Gene therapy with adenovirus vector is a promising strategy for treatment of cancer, but the antitumor effect of a single dose of adenovirus-mediated gene therapy is often insufficient in clinics (6, 19, 20), possibly due to limited transduction efficiency of adenovirus vectors. In the present study, we found that radiation dramatically enhanced adenovirus-mediated gene expression, and we clarified the mechanism of this phenomenon. In conclusion, the present data suggest that regional radiation may significantly improve adenovirus-mediated gene transfer efficiency in pancreatic tumors and probably contributes to decreasing the dose of adenovirus required for gene transfer and controlling side effects of adenovirus infection in nonirradiated normal tissue.

**References**


22. Bruening W, Giasson B, Mushynski WS, Durham HD.


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