ABSTRACT

Pancanceric cancer is one of the most lethal malignant tumors. Insulin-like growth factor (IGF)-I receptor (IGF-Ir) signaling is required for maintenance of growth and tumorigenicity of many tumors, but this pathway has not been well studied in pancreatic cancer. We have shown previously successful therapy in colorectal and lung cancer xenograft models using recombinant adenoviruses expressing dominant negative IGF-I receptors. In this study, we sought to better dissect the mechanism of action of this virus and determine whether IGF-Ir targeted adenoviruses represent potentially effective therapeutics for human pancreatic cancer cells. Truncated IGF-I receptors (IGF-Ir/dn; 482 and 950 amino acids long, respectively, IGF-Ir/482st and IGF-Ir/950st) that function as dominant negative inhibitor were cloned into recombinant adenoviruses and used to treat human pancreatic cancer cells. We assessed the effect of IGF-Ir/dn on signaling blockade, growth, stress response, chemotherapy, radiation-induced apoptosis, and in vivo therapeutic efficacy in xenografts. IGF-Ir/dn expression suppressed tumorigenicity both in vitro and in vivo and up-regulated stressor-induced apoptosis. It effectively blocked both IGF-I and IGF-II-induced activation of Akt-1. IGF-Ir/dn expression increased radiation and chemotherapy-induced apoptosis, and the combination therapy of IGF-Ir/dn with chemotherapy was very effective against tumors in mice. In an i.p. model, IGF-Ir/dn therapy reduced dissemination and prolonged survival times. Moreover, IGF-Ir/482st was more effective than IGF-Ir/950st because of its bystander effect. The antitumor activity of IGF-Ir/dn is mediated through inhibition of Akt-1 and enhances the efficacy of chemotherapy. Adenovirus-IGF-Ir/482st may be a useful anticancer therapeutic for pancreatic cancer.

INTRODUCTION

Pancreatic adenocarcinoma is one of the human cancers with the worst prognosis. Pancreatic cancer is characterized by late clinical presentation, difficulty in early diagnosis, rapid progression, and unresponsiveness to chemotherapy, radiotherapy, and immunotherapy, resulting in low resectability rates at diagnosis, early recurrence after resection, and extremely poor survival rates (1–3). Despite being a relatively infrequent tumor, pancreatic cancer ranks fourth as a cause of cancer-related mortality in Japan and the United States (1).

Signals form a variety of growth factors and their receptors affect the continuous growth potential of tumors through altered regulation of the cell cycle, apoptosis, and interactions with their environment (4). Among these are the IGFs that can stimulate cellular proliferation and induce cellular differentiation (5). The IGF-Ir is a heterodimer of two α chains and two β chains (6). Binding of the ligands, IGF-I and IGF-II, to IGF-Ir causes receptor autophosphorylation and activation of tyrosine kinase activity, which subsequently phosphorylates a host of intracellular substrates, including insulin receptor substrate-1 and Shc. These early events lead to the activation of multiple signaling pathways, such as the MAPK (also called ERK) and PI3-K/Akt-1 (protein kinase B) pathways (7, 8). In certain systems, the IGF-Ir appears to be essential for malignant transformation (9, 10). IGF-Ir is also important for the maintenance, as well as the initiation of the malignant state (9). Reduction of IGF-Ir has been shown to induce apoptosis in tumors but produce only growth arrest in untransformed cells (4). In addition, IGF-Ir knockout mice are viable (although physically much smaller than normal), indicating that relatively normal development and tissue differentiation can occur in its absence (11). These findings suggest a potential basis for tumor selectivity in therapeutic applications.

There are several possible approaches to blocking receptor signaling. One is through reduction of expression with an antisense IGF-Ir, as has been reported to suppress the tumorigenicity and caused shrinkage of established tumors (12–14). Another approach is to use dn receptors to inhibit the function rather than expression of the naturally expressed receptor. The dn receptors for IGF-Ir contain a portion of the molecule including the extracellular domain with a mutant or deleted intracellular tyrosine kinase domain, allowing nonfunctional heterodimerization (15–17). Transfection of a mutant receptor construct with only the extracellular domain resulted in the induction of differentiation and inhibition of adhesion, invasion, and metastasis, as well as demonstrating a bystander effect (18–21). We constructed adenoviruses expressing two different truncated IGF-Ir constructs (IGF-Ir/dn; a truncated 482 amino acid residue IGF-Ir/482st and the 950 amino acid residue IGF-Ir/950st). The former is a truncated extracellular domain of IGF-Ir (with neither transmembrane nor cytoplasmic domains) and thus should result in a secreted soluble form of the receptor that may affect neighboring cells in addition to the transduced cells. The latter produces intact α subunit and defective β subunit lacking the tyrosine kinase domain and was hypothesized to remain on the membrane of the transduced cells and form nonfunctional heterodimers. We reported previously that IGF-Ir/dn may be a useful therapeutic strategy for both colon and lung cancer cells and may be a more powerful strategy than antisense approach (14, 22, 23).

In this study, we evaluated the effects of IGF-Ir blockade in human pancreatic cancer cell lines. There is an extensive literature documenting the importance of the IGF signaling pathway in these tumors. Both IGF-Ir overexpression and IGF-mediated growth responsiveness are found in pancreatic cancer cells, and a monoclonal anti-IGF-Ir antibody (aIgR3) inhibits cell growth (24). IGF-I antagonizes the antipro-
liferative effects of cyclooxygenase-2 inhibitors on BxPC-3 cells (25). Thus, IGFs are thought to participate in aberrant autocrine and paracrine activation of IGF-IR in pancreatic cancer in vivo. We have assessed the impact of IGF-IR/dn on signaling blockade, growth, stressor (e.g. chemotherapy and radiation) induced apoptosis, and in vivo therapeutic efficacy in s.c. and i.p. xenografts. These observations strengthen the rationale for using soluble IGF-IR gene therapy alone or in combination with chemotherapy or radiotherapy in the targeted therapy of human pancreatic cancers.

MATERIALS AND METHODS

Materials. Antiphosphotyrosine (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Akt1 (c-20), anti-ERK1/2 (K-23), anti-phospho-ERK1/2 (E-4), anti-IGF-Ira (2C8), anti-IGF-IRβ (C20), anti-insulin Ra (N-20), and anti-phospho-p38 (A-12), anti-phospho-p38 (D-8), anti-p-Tyr (PY99) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IGF-IR (xIR-3) was purchased from Oncogene Research Products (Cambridge, MA). A P33-K inhibitor Wortmannin, a p38 MAPK inhibitor SB203580, and 5-FU were purchased from Sigma (St. Louis, MO); a MEK1 (MAPK kinase 1) inhibitor PD98059 was from New England BioLabs; and protein kinase A inhibitor was obtained from Calbiochem (San Diego, CA). Recombinant human IGF-I and IGF-II were purchased from Genzyme/Techne (Minneapolis, MN).

Construction of Recombinant Adenovirus Vectors. Recombinant adenovirus vectors were performed as described previously (22, 23). Briefly, the cDNAs of IGF-IR/482st and IGF-IR/950st were generated by reverse transcription-PCR from mRNA of National Cancer Institute H460 with primers designed for insertion of a stop codon at residues 950 and 482. Both ends of the cDNA were engineered to contain EcoRI restriction sites to clone the fragment into the polylinker site of the pAC shuttle plasmid. pAC contains the cytomegalovirus immediate early enhancer and promoter and the SV40 polyadenylation site. The entire insert was sequenced to verify the structure, and the resulting pAC-cytomegalovirus-IGF-IR/dn and vector plasmid pJM17 were cotransfected into 293 cells by standard calcium phosphate coprecipitation methods. Recombinant adenoviruses-IGF-IR/dn (Ad-IGF-IR/482st and Ad-IGF-IR/950st) were generated by homologous recombination. The resulting adenoviruses were confirmed by sequencing of insert PCR products, and plaque was purified three times. A recombinant adenovirus expressing the β-galactosidase gene was used as a control virus (Ad-LacZ).

Cell Lines and Mice. All human pancreatic cancer cell lines (AsPC-1, BxPC-3, and Panc-1) were obtained from the Japanese Cancer Collection of Research Resources (Osaka, Japan). The former two cells were passaged in RPMI 1640 and the latter in DMEM, respectively, both with 10% fetal bovine serum. Specific pathogen-free female BALB/cAnNCrj-nu mice, 6 weeks old, were purchased from Charles River Japan (Yokohama, Japan). They were maintained in a specific pathogen-free environment, and the care and use of mice were according to our university’s guidelines.

Western Blotting. Cells were treated as indicated in the text. Cell lysates were prepared as described previously (22). Protein concentration was estimated by the Bradford method (Bio-Rad). Equal aliquots of lysate (100 μg) were separated by 10% SDS-PAGE and immunoblotted onto polyvinylidene difluoride membranes. The membranes were washed, and incubated with 5% nonfat dry milk in TBST. The membranes were incubated with a 1:5000 dilution of the corresponding primary antibodies (e.g. stressor (e.g. chemotherapy and radiation) induced apoptosis, and in vivo therapeutic efficacy in s.c. and i.p. xenografts. These observations strengthen the rationale for using soluble IGF-IR gene therapy alone or in combination with chemotherapy or radiotherapy in the targeted therapy of human pancreatic cancers.

Assessment of the Effect on Tumorigenicity in Vitro. To assess the effect of truncated IGF-IR on tumorigenicity in vivo, BxPC-3 cells infected with Ad-IGF-IR/482st or Ad-LacZ were inoculated s.c. into mouse right hip region at a concentration of 2 × 10⁶ cells/mouse (6-week-old female nude mice). Tumor diameters in mice were measured with calipers, and tumor volume was calculated using the formula: tumor volume (mm³) = (width² × length)/2.

Measurement of the Effect of IGF-IR/dn on Apoptosis. Caspase-3 colorimetric protease assay was performed following the manufacturer’s protocol (Caspase-3 Colorimetric Protease Assay Kit; MBL). In brief, 5 × 10⁶ cells were lysed in 250 μl of chilled cell lysis buffer, and total cell lysates (100 μg) were incubated with 4 μM VETD-pNA Substrate (200 μM final concentration) at 37°C for 1 h. Caspase-3 activity was measured by colorimetric reaction at 400 nm.

DNA fragmentation assay was performed as follows: low molecular weight DNA was extracted with 0.5% Triton X-100, 10 nM EDTA, and 10 mM Tris-HCl (pH 7.4); treated with 400 μg/ml RNase A and Proteinase K for 1 h at 37°C; ethanol precipitated; and subjected to 1% agarose gel electrophoresis. The gels were stained with 1 μg/ml ethidium bromide.

First, PANC-1 and BxPC-3 cells infected Ad-IGF-IR/dn or Ad-LacZ were induced with serum starvation and 5% ethanol and assayed with caspase-3 assay and DNA fragmentation assay. To assess the effect of IGF-IR/dn on chemotherapy inducing apoptosis, tumor cells were treated with 260 μM 5-FU, and apoptosis was assessed using caspase-3. To assess the effect of truncated IGF-IR on radiation, the cells were treated with radiation (30 Gy, 11 min) and then measured by caspase-3 assay. To identify apoptosis in tumors on mice, TUNEL assays were performed following the manufacturer’s protocol (in situ labeling and detection system; DAKO).

Assessment of in Vivo Therapeutic Efficacy in Established Tumors. We assessed the effect of truncated IGF-IR on established tumors in mice. Two × 10⁶ BxPC-3 cells were s.c. injected into 10 nude mice in the first series and 12 in the second. After all s.c. tumors were palpable (20 days after inoculation), 2 × 10⁶ PFU of Ad-IGF-IR/482st or Ad-LacZ were injected into the tumors daily for five consecutive days. Mice were euthanized when tumors reached 2 cm in size or the data were clinically evident symptoms. Formalin-fixed paraffin sections were used for TUNEL assays.

Then we examined an additional effect of Ad-IGF-IR/482st on chemotherapy of established tumors. Two × 10⁶ BxPC-3 cells were s.c. injected into 24 nude mice. After all s.c. tumors were palpable (20 days after inoculation), 2 × 10⁶ PFU of Ad-IGF-IR/482st or Ad-LacZ were injected into the tumors daily for five consecutive days. Both groups were then divided into pair-matched chemotherapy treatment and control groups (n = 6, 5-FU (50 mg/kg i.p.), and vehicle was administered i.p., and this was repeated once per week, for five total doses. Tumor diameters were serially measured, and tumor volume was calculated.

Assessment of in Vivo Therapeutic Efficacy in Peritoneal Dissemination Model. To assess the effect of truncated IGF-IR on peritoneally disseminated tumors in mice, 1 × 10⁸ BxPC-3 cells were injected into the peritoneal cavities of 28 nude mice. Three days after cancer cell injection, 50 m.o.i. (based on the injected tumor cell number) of Ad-IGF-IR/482st or Ad-LacZ were injected into the peritoneal cavity daily for five consecutive days. Five mice in each group were euthanized on day 27, in which the first mouse appeared lethargic in a pilot study. Other mice were carefully observed everyday until they died.

Statistical Analysis. The results are presented as means ± SE for each sample. The statistical significance of differences was determined by Student’s two-tailed t test in two groups and done by one-way ANOVA in multiple groups and two-factor factorial ANOVA. Survival curve was constructed according to the method of Kaplan and Meier. P ≤ 0.05 was considered to indicate statistical significance. All data were calculated with a Statview 5.0 (Abacus Concepts, Berkeley, CA) statistical software package run on a Macintosh personal computer (Apple computer, Cupertino, CA).
RESULTS

Adenoviral Induction of the Truncated IGF-Ir cDNA into Pancreatic Cancer Cells. Adenoviruses expressing the two different IGF-Ir/dn cDNAs were constructed. Production of IGF-Ir/dn after transduction with the adenoviruses (m.o.i. 10–100) was confirmed by fluorescence-activated cell sorter analysis (Fig. 1a) and Western blotting (Fig. 1b). IGF-Ir/950st was detected on the cell surface of three pancreatic cell lines, PANC-1, BxPC-3, and AsPC-1, by fluorescence-activated cell sorter analysis. The amount of the receptor was increased in accordance with that of adenovirus. IGF-Ir is not detected usually in the culture medium because the receptor is membrane bound. IGF-Ir/482st was detected in the culture media by Western blotting, but not on the cell surface, because this truncated form does not have a transmembrane domain. The presence of the soluble truncated receptor in the medium suggested the possibility of a bystander effect.

In Vitro Growth Is Dramatically Inhibited in Cells Producing IGF-Ir/482st. To evaluate the effect of Ad-IGF-Ir/dn on in vitro cell growth, trypan blue dye exclusion assays were performed on pancreatic cancer cell lines, PANC-1 and BxPC-3, 24, 48, and 72 h after recombinant adenovirus infection. Although control cells infected with Ad-LacZ continued to grow, cells infected with Ad-IGF-Ir/482st steadily decreased (PANC-1, \( P = 0.0014 \); BxPC-3, \( P = 0.0037 \); Fig. 2).

Serum Starvation- and Ethanol-induced Apoptosis Are Increased by Ad-IGF-Ir/dn. BxPC-3 cells were stressed by serum starvation (24 h) or 5% ethanol (1 h) after adenoviral infection and then analyzed induced apoptosis using caspase-3 colorimetric protease assay and DNA fragmentation assay. Five percent ethanol upregulated caspase-3 activity of Ad-LacZ-infected BxPC-3 cells 1.57 ± 0.06 times (\( P = 0.0004 \); Fig. 3a). Both IGF-Ir/482st and IGF-Ir/950st enhanced ethanol-induced apoptosis (2.27 ± 0.13 times)
and 2.02 ± 0.11 to the cells infected with control virus without ethanol stimulation, respectively; P < 0.0001 and P = 0.0399 compared with the cells infected with control and stimulated by ethanol, respectively; Fig. 3a), which was confirmed by DNA fragmentation assay (Fig. 3b). The same result was detected in AsPC-1 cells (Fig. 3c). IGF-Ir/482st proved to be more potent than IGF-Ir/952st in the caspase-3 assay, so we used Ad-IGF-Ir/482st mainly in the following experiments. Serum starvation-induced apoptosis in BxPC-3 cells infected with Ad-LacZ was, however, not statistically significantly different, indicating that serum starvation alone is not an efficient inducer of apoptosis (Fig. 3d). IGF-Ir/482st enhanced serum starvation-induced apoptosis to 1.79 ± 0.13 times (P = 0.0035 compared with the cells infected with control virus and starved; P = 0.0023 versus the cells infected with Ad-IGF-Ir/482st cultured with complete media; Fig. 3d).

Both Chemotherapy and Radiation-induced Apoptosis Are Increased by IGF-Ir/dn. BxPC-3 cells were treated with the chemotherapy agent 5-FU or radiation after infection with adenoviruses. 5-FU (250 μM) induced caspase-3 activity of the BxPC-3 cells infected with control vector 1.73 ± 0.04 times compared with the cells without 5-FU (P < 0.0001; Fig. 3e). In the presence of IGF-Ir/482st, the activity was increased to 2.01 ± 0.1 times (P = 0.0248 compared with the cells with control virus and the same stimulation; Fig. 3e). Radiation also induced caspase-3 activity of the cells infected with control virus 1.53 ± 0.07 times compared with that without radiation (P < 0.0001; Fig. 3f). IGF-Ir/482st up-regulated radiation-induced apoptosis, 1.74 ± 0.05 times (P = 0.0385 compared with the control cells with radiation; Fig. 3f).

The Truncated IGF-Ir Blocks IGF’s Signaling through Akt-1. In both BxPC-3 and PANC-1 cells, Akt-1 was clearly phosphorylated with IGF-I between 10 and 100 ng/ml and with IGF-II between 5 and 50 ng/ml, and this phosphorylation was observed from 5 to 25 min after treatment with ligands (data not shown). The effect of 20 ng/ml IGF-I on Akt phosphorylation was equivalent to that of 10 ng/ml IGF-II, so these ligands were used at these concentrations for additional experiments. IGF-induced phosphorylated Akt was reduced by the infections of both Ad-IGF-Ir/482st and Ad-IGF-Ir/950st. Between m.o.i. 30 and m.o.i. 100, both adenoviruses showed almost the same effect on the blockade of Akt signaling (Fig. 4a). Akt-kinase assay showed that m.o.i. 10 of Ad-IGF-Ir/482st was not sufficient to block phosphorylation of Akt and m.o.i. 30 of which blocked it (Fig. 4b), so we used m.o.i. 30 in the following experiments. Phosphorylation of ERK-1 and -2 was influenced to a much lesser degree than Akt-1 in both cells infected with Ad-IGF-Ir/dn receptors (Fig. 4c). In both AsPC-1 and BxPC-3 cells, Ad-IGF-Ir/dn also blocked IGF-I inducing phosphorylation of p38 (Fig. 4d).

In both AsPC-1 and BxPC-3 cells, des(1–3)IGF-I could phosphorylate Akt-1, however, IGF-Ir/482st blocked des(1–3)IGF-I-induced phosphorylation of Akt (Fig. 4e). This suggests that the effect of IGF-Ir/dn is not likely to be a consequence of altered levels of the IGFBPs in these cell lines.

To investigate signaling pathways downstream from IGF-Ir, three inhibitors were used. The effects of these inhibitors on signal blockade in BxPC-3 cells were studied using Western blot assay. The PI3-K inhibitor Wortmannin (200 nM) completely inhibited Akt phosphorylation, the MEK1 inhibitor PD98059 (10 μM) suppressed completely phosphorylated ERK-1, and the p38 inhibitor SB203580 (20 μM) blocked P38 phosphorylation almost fully (data not shown). Both Wortmannin and PD98059 reduced the in vitro cell number of both AsPC-1 and BxPC-3 cells as effectively as Ad-IGF-Ir/482st; however, SB203580 did not influence cell growth (Fig. 5, a and b). Combined with the fact that IGF-Ir/dn blocked IGF-induced phosphorylation of Akt and p38 but not ERK, these data suggest that the antimitogenic effect of IGF-Ir/dn is mediated by the inhibition of PI3-K/Akt in pancreatic cancer cells.

Both Wortmannin and SB203580 up-regulated 5% ethanol-induced apoptosis in BxPC-3 cells, but PD98059 did not influence apoptosis (Fig. 5, c–e). In combination with the result that IGF-Ir/dn blocked phosphorylation of Akt and p38 but not ERK, this suggests that both the PI3-K/Akt and p38 pathways appear to mediate the antiproliferative effect of the IGFs in human pancreatic tumor cells.

The Short Form of IGF-Ir Has a Bystander Effect. BxPC-3 cells were infected with Ad-IGF-Ir/482st or Ad-LacZ, after being washed several times with PBS to remove residual input adenovirus, and subsequently cultured with complete medium for several hours. This conditioned medium was then transferred to untreated cultures of BxPC-3 cells. Trypan blue dye exclusion assays were performed on noninfected BxPC-3 cells cultured with conditioned medium to evaluate in vitro cell growth. Conditioned medium from cells infected with Ad-IGF-Ir/482st suppressed cell growth significantly compared with that from a control virus infection (P = 0.0122; Fig. 6a). Caspase-3 activity was measured in noninfected BxPC-3 cells cultured with conditioned medium after treatment with 5% ethanol. The media of the cells infected with Ad-IGF-Ir/482st resulted in increased apoptosis compared with control virus (1.41 ± 0.05 and 1.69 ± 0.09, respectively, P = 0.0225; Fig. 6b). However, the effect of conditioned media cultured from cells infected with Ad-IGF-Ir/950st on both growth inhibition and promotion of apoptosis in untreated BxPC-3 cells is similar to those of control virus (data not shown). These results demonstrate a bystander effect and suggest that gene therapeutic strategies using human IGF-Ir/482st should have enhanced antitumor effects.
The Soluble Receptor Virus Inhibits in Vivo Tumorigenicity, and the Combination of IGF-Ir/482st and Chemotherapy Causes Regression of Established Tumors. We then evaluated the effects of receptor blockade in vivo. At the tumor inocula used, the incidence of s.c. tumors derived from BxPC-3 cells infected with Ad-LacZ and those with Ad-IGF-Ir/482st were 100%, but PANC-1 cells did not form any tumors. The growth of s.c. tumors derived from the BxPC-3 cells expressing IGF-Ir/dn showed dramatic differences, however (the cells with control versus those with IGF-Ir/482st, \( P = 0.0399 \) in the first series and \( P < 0.0001 \) and 0.0399, respectively, compared with ethanol-induced apoptosis in the cells infected with control virus). In b and c, DNA fragmentation assay shows that ethanol-inducing apoptosis apparently in the short receptor expressing cells (Lane 4). Both BxPC-3 (b) and AsPC-1 (c) cells are infected with Ad-LacZ (Lanes 1 and 2) or Ad-IGF-Ir/482st (Lanes 3 and 4) and are stimulated with 5% ethanol (Lanes 2 and 4) or are not stimulated (Lanes 1 and 3). d, caspase-3 assay demonstrates that 24 h of serum starvation is not sufficient for induction of apoptosis. Truncated receptor up-regulates serum starvation inducing apoptosis markedly (\( P = 0.0023 \) compared with starved cells infected with control virus). CM, complete medium; SFM, serum-free medium. Both 260 \( \mu \)M 5-FU (e) and radiation (30 Gy; f) induce apoptosis by caspase-3 assay (\( P < 0.0001 \)). IGF-Ir/482st up-regulates the effects of both treatment on apoptosis induction (\( P = 0.0248 \) and 0.0385, respectively). Bars show means and SE.

![Image](https://example.com/image.png)

Fig. 3. IGF-Ir/dns enhance apoptotic responses to 5% ethanol, serum starvation, chemotherapy, and radiotherapy, in BxPC-3 (a, b, d, e, and f) and AsPC-1 (c) cells. In a, caspase-3 calorimetric protease assay demonstrates apoptosis induction by 5% ethanol (\( P = 0.0004 \)). IGF-Ir/482st expression alone induces apoptosis (\( P = 0.0007 \)). Both IGF-Ir/dns up-regulate ethanol-inducing apoptosis, and IGF-Ir/482st is more effective than IGF-Ir/950st (\( P < 0.0001 \) and 0.0399, respectively, compared with ethanol-induced apoptosis in the cells infected with control virus). In b and c, DNA fragmentation assay shows that ethanol-inducing apoptosis apparently in the short receptor expressing cells (Lane 4). Both BxPC-3 (b) and AsPC-1 (c) cells are infected with Ad-LacZ (Lanes 1 and 2) or Ad-IGF-Ir/482st (Lanes 3 and 4) and are stimulated with 5% ethanol (Lanes 2 and 4) or are not stimulated (Lanes 1 and 3). d, caspase-3 assay demonstrates that 24 h of serum starvation is not sufficient for induction of apoptosis. Truncated receptor up-regulates serum starvation inducing apoptosis markedly (\( P = 0.0023 \) compared with starved cells infected with control virus). CM, complete medium; SFM, serum-free medium. Both 260 \( \mu \)M 5-FU (e) and radiation (30 Gy; f) induce apoptosis by caspase-3 assay (\( P < 0.0001 \)). IGF-Ir/482st up-regulates the effects of both treatment on apoptosis induction (\( P = 0.0248 \) and 0.0385, respectively). Bars show means and SE.

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TUNEL-positive cells, the number of apoptotic cells was significantly increased in tumors injected with Ad-IGF-Ir/482st compared with controls, 35 ± 5.5 versus 8.2 ± 8.2, respectively (P = 0.0383; Fig. 7c). The data indicate that shorter form of the receptor effectively induced apoptosis in vivo.

As IGF-Ir/dn enhanced 5-FU-induced apoptosis in vitro, the effect of combined IGF blockade with chemotherapy on tumors in mice was then assessed. Established s.c. BxPC-3 tumors were intratumorally injected both adenoviruses (1 × 10^8 PFU, half the dose used in the previous experiments) for 5 days, and then the mice were injected i.p. with 5-FU (50 mg/kg) or PBS once a week for five doses. 5-FU significantly reduced the size of tumor and suppressed growth (Ad-LacZ/PBS versus Ad-LacZ + 5-FU, P = 0.0002; Fig. 7d). The tumors were shrunk by the presence of IGF-Ir/482st, and the growth was reduced (Ad-LacZ + PBS versus Ad-IGF-Ir/482st + PBS, P < 0.0001), as same as previous experiments (22). The combination therapy of Ad-IGF-Ir/482st and 5-FU was the most effective for the tumors derived from BxPC-3 (Ad-LacZ + PBS versus Ad-IGF-Ir/482st + 5-FU, P = 0.0005; Fig. 7d).
482st + 5-FU, \( P < 0.0001 \); Fig. 7d), and two of six tumors disappeared completely (Table 3). This indicates that Ad-IGF-Ir/482st has potential to enhance other strategies for the therapy of pancreatic cancer, including chemotherapy.

**Ad-IGF-Ir/482st Reduced Peritoneal Dissemination and Prolonged Survival.** Although neither PANC-1 nor BxPC-3 cells formed lymph node and distant metastases after i.p. injection in our pilot study, AsPC-1 cells have been reported to do this (26). AsPC-1-bearing mice were treated by the i.p. administration of Ad-IGF-Ir/482st or control virus. Ad-IGF-Ir/482st reduced the number of lymph node metastases \( (P = 0.0007) \) and, moreover, up-regulated 5% ethanol-inducing apoptosis \( (P = 0.0013) \) in BxPC-3 cells. SB203580 enhanced ethanol-inducing apoptosis \( (e, P = 0.0019) \), but PD98059 did not \( (d) \). NS, not significant.

**DISCUSSION**

The clinical course of human pancreatic adenocarcinoma is generally characterized by an advanced stage at the time of diagnosis, a high frequency of lymph node metastasis, peritoneal dissemination, and hepatic involvement, and a paucity of meaningful treatment strategies. IGF-mediated growth responsiveness is found in most cancer cells of epithelial origin, including pancreas (24, 27). Recent studies have shown that elevation of serum IGF-I increases the risk of developing several cancers (28–30). In pancreatic cancer, aberrant activation of IGF-Ir by paracrine and autocrine mechanisms has been suggested because IGF-I mRNA expression is increased in tumor tissue (24). The functional importance of IGF-Ir in pancreatic cancer was demonstrated by the addition of antisense IGF-Ir, which decreased basal and IGF-I-stimulated cell growth of AsPC-1 (24). In the current study, we developed adenoviral dn receptor systems for the accurate dissection of the responsible signaling pathways and evaluated the effect of controlled and selective IGF-Ir blockade. They
used media for cells infected with Ad-LacZ.

Inducing apoptosis.

The initial proliferative switch is correlated with the IGF-II, involved in the autocrine growth stimulation of pancreatic tumor cells does not seem to favor tumor spread (27). However, IGF-II is involved in the in vivo suppressed tumorigenicity both in vivo and in vitro and enhanced chemotherapy- and radiation-induced apoptosis in vitro and chemotherapeutic efficacy in vivo. Those results indicated that this strategy is promising for treatment of pancreatic cancer as it works well alone and moreover potentiates chemotherapy and radiotherapy. Resistance to chemotherapy or radiotherapy is a serious problem in cancer treatment, and this approach has a potentiality for overcoming.

IGF-I levels are increased in pancreatic cancer patients, but this does not seem to favor tumor spread (27). However, IGF-II is involved in the autocrine growth stimulation of pancreatic tumor cells (31, 32). The initial proliferative switch is correlated with the IGF-II, and an antisense IGF-II interferes with tumor cell proliferation in vitro. Tumors in IGF-II knockout mice were reduced malignancy and enhanced chemotherapy- and radiation-induced apoptosis of noninfected cells. Of these two, IGF-I levels are increased in pancreatic cancer patients, but this does not seem to favor tumor spread (27).

Two different truncated IGF-Ir mutants were studied in this study, IGF-Ir/482st and IGF-Ir/950st. Genetic blockade of IGF signaling with two forms of IGF-Ir/dn dramatically suppresses tumorigenesis and promotes apoptosis, both in vitro and in vivo. Of these two, IGF-Ir/482st increased apoptosis more effectively than IGF-Ir/950st, which confirms our previous report that Ad-IGF-Ir/482st had a greater antitumor effect than Ad-IGF-Ir/950st against human colon cancer xenografts in mice (22). The main reason for this difference may be that IGF-Ir/482st produces a demonstrable bystander effect, blocking IGF-II-mediated signaling even in non-gene-modified cells. We demonstrated direct evidence for a bystander effect in this study by the fact that soluble receptor-containing culture media reduced proliferation and up-regulated ethanol-induced apoptosis of noninfected cells. In contrast to approaches requiring plasmid transfection, adenoviral...

Fig. 6. Ad-IGF-Ir/482st shows bystander effects. After washing, BxPC-3 cells, which were infected with 30 m.o.i. of Ad-IGF-Ir/482st or control, were cultured with complete media, and then those media were collected. To analyze the bystander effects, BxPC-3 cells without any infection were cultured with those collected media. In a, in vitro cell growth was suppressed by those collected media; the media of cells infected with Ad-IGF-Ir/482st (○) reduced the number of cells compared with that with Ad-LacZ (□); P = 0.0122. In b, caspase-3 assay revealed that 5% ethanol inducing apoptosis (P = 0.0006) in BxPC-3 cells cultured with used media for cells infected with control virus. The media used for cells infected with Ad-IGF-Ir/482st up-regulated ethanol inducing apoptosis. P = 0.0225 compared with ethanol-stimulating cells cultured with used media for cells infected with Ad-LacZ.

Fig. 7. a, BxPC-3 cells infected with Ad-IGF-Ir/482st (●) or Ad-LacZ (□) before s.c. inoculation into nude mice. The panel shows the second series of experiments (n = 6/group). There were significant differences in the tumor sizes among these groups (P = 0.0011). b, in independent experiments, established palpable s.c. BxPC-3 tumors were directly injected with 2 × 10⁶ PFU of Ad-IGF-Ir/482st (●) or Ad-LacZ (□) on five successive days, and the relative tumor volumes were measured. This is the result of a second experiment (n = 6/group). Ad-IGF-Ir/dn blocked tumor growth markedly (P = 0.0002), and two of six tumors disappeared completely. In c, the number of apoptotic cells in s.c. tumor by TUNEL staining was counted in 20 (× 200) fields per slide 39 days after injections of virus into established tumors (first experiment, n = 5/group). Truncated receptor increased in vivo induction of apoptosis (P = 0.0382). In d, combination therapy of 5-FU and Ad-IGF-Ir/482st was assessed. After forming s.c. BxPC-3 tumors, 1 × 10⁸ PFU of Ad-IGF-Ir/482st (●) or Ad-LacZ (□) were directly injected on five successive days, and 5-FU (dotted line) or vehicle (PBS, solid line) was injected (i.p.) five times, once per week. Both 5-FU alone (○ and dotted line) and IGF-Ir/482st alone (● and solid line) suppressed tumor growth (P = 0.0002 and <0.0001, respectively). The combined therapy (● and dotted line) was the most effective, and two of six s.c. tumors were cured (P < 0.0001 compared tumor treated with control virus and PBS).
DOMINANT NEGATIVE IGF-I FOR PANCREATIC CANCER

The bioactivity of IGF-I is known to be modulated by several high affinity soluble proteins, IGFBP (7, 28). These IGFBPs control the distribution of IGF-I between the extracellular environment and cell surface binding sites and can alter IGF bioactivity by modulating its interaction with the receptor. In this study, des(1–3)IGF-I, an analogue with reduced affinity to IGFBPs, resulted in effective activation of Akt-1, and the expression of IGF-Ir/dn blocked the des(1–3)IGF-I-inducing phosphorylation of Akt. This result indicates that the effect of IGF-Ir/dn is not significantly affected by IGFBPs in these pancreatic cancer cells.

In this study, we are the first to show that peritoneal growth and metastasis of pancreatic cancer was reduced by the treatment with Ad-IGF-Ir/482st, and this resulted in improved survival. Previous studies have shown that cancer cells adhere to peritoneal mesothelium on days 5–7 and start proliferating and infiltrating the muscle layer on days 9–11 after i.p. inoculation (42, 43). However, mice treated with Ad-IGF-Ir/dn from 5 to 9 days after inoculation in this study showed substantially reduced implantation and invasion. The peritoneal dissemination and locoregional recurrence are the most frequent modes of recurrence after resection of pancreatic cancer (44). Therefore, prevention and treatment for peritoneal dissemination are important therapeutic end points for the development of pancreatic cancer therapeutics, and our studies demonstrate that Ad-IGF-Ir/482st is very potent in this effect.

IGF-Ir/dn expression suppressed tumorigenicity and up-regulated vectors are a much more efficient and practical method for delivering therapeutic genes to cancer cells, particularly when the transduced gene has a potent bystander effect. Thus, strategies such as these targeting IGF-Ir may become practical and effective cancer therapeutics for pancreatic adenocarcinoma.

The downstream signaling pathways responsible for the observed effects of the IGFs are a matter of intense investigation. Both Akt and MAPKs (ERK-1, -2, and p38) are highly activated during progression of pancreatic carcinoma (35–39). We demonstrated that both Wortmannin (PI3-K inhibitor) and SB203580 (p38 inhibitor) up-regulated ethanol-induced apoptosis to a similar degree as IGF-Ir/dn; however, PD98059 (MEK inhibitor) did not, which indicated that IGF-Ir/dn blocks mainly IGF-derived antiapoptotic signals through PI3-K/Akt-1 and p38 in pancreatic cancer cells. Previous reports have demonstrated that there are PI3-K/Akt-dependent and -independent survival signaling pathways used by IGF-I (40). Increased resistance of MIA PaCa-2 cells to apoptosis by IGF-Ir stimulation was mediated through MAPK and PI3-K pathways and as yet unidentified pathway(s) (37). Thus, the influence of IGF-Ir/dn on the third pathway has to be investigated and the p38 is suggested by this study to be a good candidate. We also found that SB203580 did not alter cell growth, which confirms previous reports that SB203580 did not alter the mitogenicity of IGF-I in pancreatic cancer cell lines (AsPC-1, COLO-357, PANC-1, and T3M4; Ref. 41). However, both Wortmannin and PD98059 inhibited in vitro growth to the same degree as IGF-Ir/dn. The fact that IGF-Ir/dn blocked phosphorylation of Akt-1 and p38, but not ERK-1/-2, indicated that IGF-Ir/dn blocks IGF-derived growth signals through the PI3-K/Akt-1 pathway. This strategy is further supported by the published evidence that there is a cross-talk between IGF-Ir and Akt-1, which up-regulates IGF-I expression and increases invasiveness (38).

The Table 1 Summary of s.c. tumors derived from adenovirus-infected BxPC-3 cells on nude mice

<table>
<thead>
<tr>
<th>Intratumor injection</th>
<th>Ad-LacZ</th>
<th>Ad-IGF-Ir/482st</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative tumor volume</td>
<td>57.3 ± 24.9</td>
<td>57.3 ± 24.9</td>
</tr>
<tr>
<td>Disappearance of tumor</td>
<td>2nd series (n = 6)</td>
<td>2nd series (n = 6)</td>
</tr>
<tr>
<td>Relative tumor volume</td>
<td>77.0 ± 16.1</td>
<td>77.0 ± 16.1</td>
</tr>
<tr>
<td>Invasion to the muscle layer</td>
<td>2nd series</td>
<td>2nd series</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>29.1 ± 4.1</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td>Invasion to the muscle layer</td>
<td>1st series</td>
<td>1st series</td>
</tr>
<tr>
<td>Relative tumor volume</td>
<td>119.7 ± 318.9</td>
<td>119.7 ± 318.9</td>
</tr>
<tr>
<td>Disappearance of tumor</td>
<td>1st series (n = 5)</td>
<td>1st series (n = 5)</td>
</tr>
</tbody>
</table>

Table 2 Summary of treatment with adenovirus for established s.c. BxPC-3 tumors

<table>
<thead>
<tr>
<th>Intratumor injection</th>
<th>Intratumor infection</th>
<th>PBS</th>
<th>5-FU</th>
<th>PBS</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative tumor volume</td>
<td>24.9 ± 3.6</td>
<td>11.2 ± 1.1</td>
<td>9.6 ± 2.8</td>
<td>1.6 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Disappearance of s.c. tumor</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Invasion to the muscle layer</td>
<td>6 (100%)</td>
<td>4 (67%)</td>
<td>1 (17%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 Summary of combination therapy for established s.c. BxPC-3 tumors (n = 6)

<table>
<thead>
<tr>
<th>Intratumor injection</th>
<th>Ad-LacZ</th>
<th>Ad-LacZ</th>
<th>Ad-IGF-Ir/482st</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative tumor volume</td>
<td>57.3 ± 24.9</td>
<td>57.3 ± 24.9</td>
<td></td>
</tr>
<tr>
<td>Disappearance of tumor</td>
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<td></td>
</tr>
<tr>
<td>Invasion to the muscle layer</td>
<td>2nd series</td>
<td>2nd series</td>
<td></td>
</tr>
<tr>
<td>Tumor volume</td>
<td>29.1 ± 4.1</td>
<td>4.2 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Invasion to the muscle layer</td>
<td>1st series</td>
<td>1st series</td>
<td></td>
</tr>
<tr>
<td>Relative tumor volume</td>
<td>119.7 ± 318.9</td>
<td>119.7 ± 318.9</td>
<td></td>
</tr>
<tr>
<td>Disappearance of tumor</td>
<td>1st series (n = 5)</td>
<td>1st series (n = 5)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 8. In a, AsPC-1 cells disseminated after i.p. injection. i.p. injection with Ad-IGF-Ir/482st on five successive days suppressed it, but Ad-LacZ, did not. Arrows, lymph node metastasis. In b, IGF-Ir/dn reduced the numbers of lymph node metastasis (P < 0.0001) and tended to reduce live metastasis. NS, not significant. In c, IGF-Ir/482st prolonged survival time of AsPC-1 disseminated mice (P = 0.0323). ∅, Ad-LacZ; ●, Ad-IGF-Ir/482st.
apoptosis through blocking activation of Akt-1. IGF-IR/dn potentiates radiation and chemotherapy and reduces i.p. dissemination and prolonged survival time of those mice. Thus, adoviro virus IGF-IR/482st may be a promising anticancer therapeutic for pancreatic cancer.

REFERENCES

Genetic Blockade of the Insulin-like Growth Factor-I Receptor: A Promising Strategy for Human Pancreatic Cancer

Yongfen Min, Yasushi Adachi, Hiroyuki Yamamoto, et al.


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