Targeting the C-Met Receptor with a Novel Oncolytic Adenovirus Increases Infection Efficiency and Oncolytic Activity against Human Pancreatic Adenocarcinoma Cells

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Abstract

Introduction: Virotherapy using oncolytic adenoviruses (Ads) is an innovative approach to cancer therapy. The c-Met receptor and its ligand, hepatocyte growth factor (HGF), are involved in a wide array of neoplastic diseases, including pancreatic cancer. We created a novel oncolytic adenovirus by incorporating NK2, a competitive antagonist of the HGF/c-Met association, into the Ad serotype 5 knob fiber. We hypothesized that this new oncolytic Ad would result in efficient viral transduction and cell killing in an array of human pancreatic cancer cell lines.

Methods: Human pancreatic cancer lines PANC-1, CFPAC-1, AsPC-1, and BxPC-3 were obtained from the American Type Culture Collection. The efficiency of Ad infection in the cells was determined using a vector construct expressing the red fluorescence protein (RFP) marker fused to the pIX capsid gene and quantified by flow cytometry. Oncolytic activity in the pancreatic cancer cells was determined using the oncolyticAd5-pIX-RFP-FF-NK2 by a crystal violet staining method.

Results: c-Met was overexpressed in all four pancreatic cancer cell lines. The oncolyticAd5-pIX-RFP-FF-NK2 was more efficient than an untargeted wild-type Ad in transducing pancreatic cancer cell lines as well as cell killing than wild-type adenoviruses.

Conclusion: The novel recombinant oncolyticAd5-pIX-RFP-FF-NK2 efficiently transduced and killed human pancreatic cancer cells. This novel construct represents a potential new therapy for pancreatic cancer.

INTRODUCTION

Adenocarcinoma of the pancreas is a formidable disease. In the United States, it ranks fourth as the leading cause of cancer-related deaths [1]. It is estimated that in 2013, 45,220 new cases of pancreatic cancer will be diagnosed in the United States, the majority of which (85%) is adenocarcinoma arising from the ductal epithelium [1]. Pancreatic adenocarcinoma is one of the most aggressive cancers and despite advances in the perioperative care, surgical techniques, and modern chemotherapy, significant improvement in prolonging survival has been marginal [1-3]. Surgery remains the only treatment for potential cure, but even so, only a minority of patients can be considered to be truly cured [4]. Unlike breast, colon, rectal, and esophageal cancer, marginal improvement has been made for pancreatic cancer; from 1975 to 2008, the five-year survival rate for breast cancer increased from 75% to 90%, whereas for pancreatic carcinoma, it was from 2% to 6% [1]. Significant impact on this disease will, therefore, require novel therapeutics and a better understanding of its biology.

The MET proto-oncogene encodes for c-Met, a tyrosine kinase receptor, which is widely expressed in cells of epithelial-endothelial origin. The only known ligand of c-Met, is hepatocyte growth factor (HGF) found mainly in mesenchymal cells, although some epithelial cancer cells also express both c-Met and HGF.
and HGF [5]. The c-Met protein is necessary for embryonic development and tissue repair [6-8]. In response to binding of HGF, the HGF/c-Met pleiotropic signaling cascade activates mediators of cell proliferation and motility. The HGF/c-Met axis can mediate induction of tumorigenesis via activating mutation, gene amplification, and/or overexpression of c-Met in a variety of cancers including breast, lung, ovary, colon, kidney, thyroid, liver, stomach, and pancreas [9,10]. The c-Met appears to play a role in the pathogenesis of pancreatic adenocarcinoma. Histopathological analyses demonstrated that c-Met protein overexpression in pancreatic cancer increases five to seven-fold compared to normal pancreas sample [11,12]. The c-Met expression levels correlated with tumor grade in a study of 36 pancreatic tumor samples [13]. Patients whose tumors express high expression of HGF or c-Met had a significantly worse disease-free survival compared to patients without an expression of these proteins [14]. Given its role in the development of cancer, c-Met can be exploited as a potential target for the development of novel target-specific therapy.

Adenovirus(Ads) as oncolytic virotherapy is a promising therapeutic modality against a wide variety of neoplasms, including pancreatic adenocarcinoma [15]. In principle, the ideal oncolytic Ads should replicate in tumor cells, lyse the cells, and finally release viral progeny so that they can further infect adjacent tumor cells while sparing normal tissues. The serotype 5 (Ad5, subgroup C) is the most frequently used serotype in human clinical gene therapy trials [16]. However, Ad5 serotype alone is nonspecific, and transduction into tumor cells is suboptimal, the latter due to the low expression of the receptors (human Coxsackie Adenovirus Receptor or hCAR) on tumors, receptors that are required for viral entry. An alternative method of improving viral transduction is to search for an hCAR-independent pathway, a pathway that exploits a particular characteristic of the tumor. Because pancreatic cancer overexpresses c-Met, we chose to create a novel chimeric Ad5 virus by incorporating NK2, a competitive antagonist of the HGF/c-Met association, into the Ad serotype 5 knob fiber(Ad5-pIX-RFP-FF-NK2). In this study, we present the in vitro data to demonstrate the utility of using this novel viral construct to preferentially transduce and lyse human pancreatic cancer cells.

**MATERIALS AND METHODS**

**Cell lines and culture conditions**

The human pancreatic cancer lines CFPAC-1, PANC-1, AsPC-1, and BxPC-3, were obtained from the American Type Culture Collection. CFPAC-1 was cultured in Iscove’s MDM (Invitrogen; Carlsbad, CA). PANC-1 was cultured in Dulbecco’s Modified Eagle Medium (Invitrogen), and AsPC-1 and BxPC-3 were cultured in RPMI-1640 medium (Thermo Fisher Scientific; Pittsburgh, PA). All media contained 10% fetal bovine serum (FBS; Gemini BioProducts; Woodland, CA) and 1% antibiotic-antimycotic solution (Sigma-Aldrich; St. Louis, MO). The cells were maintained at 37°C in a 5% humidified CO2 incubator and were subcultured using 1% trypsin-EDTA (Invitrogen).

**Adenoviral vectors**

We replaced the native Ad5 fiber with a chimeric fiber by fusion of the N-terminus tail of the Ad5 fiber with the T4-phage fibritin protein linked with the HGF/NK2 isoform at the carboxyl (C) terminus. In brief, an Xbal-Swal fragment encompassing the chimeric Ad fiber gene was synthesized (Genscript; Piscataway, NJ) and used to replace the Xbal-Swal fragment of the wild-type Ad sequence within the pAdEasy-1 plasmid (Agilent Technologies; Santa Clara, CA), introducing the T4 phage rod-like trimeric fibritin molecule. Ampicillin-resistant colonies were selected following transformation. DNA was extracted, and identities of positive clones were confirmed by restriction digestion and PCR. A pIX-RFP reporter gene was introduced into the aAdEasy-1 by homologous recombination [17] with a modified pShuttle vector containing a wild-type Ad5 E1A gene and the mCherry coding sequence inserted downstream of the Ad5 minor capsid pIX gene to generate a C-terminal pIX fusion protein (pShuttle-E1A-pIX-RFP), a kind gift from Dr. Anton Borovjagin (University of Alabama at Birmingham, Birmingham, AL). Recombinants were selected on kanamycin agar plates and confirmed by restriction digestion and PCR. DNA sequencing was done to confirm the identity of the inserted fragments (Figures 1,2).

**Rescue, propagation, and purification of Ad virions**

As described previously [18], the genome of the fiber-modified virus was used to transfect 293/F28 cells that stably express the Ad5 wild-type fiber-expressing, by CaPO4 co-precipitation. To obtain a homogenous population of Ad5-pIX-RFP-FF-NK2virions, we replaced the native Ad5 fiber with a chimeric fiber by fusion of the N-terminus tail of the Ad5 fiber with the T4-phage fibritin protein linked with the HGF/NK2 isoform at the carboxyl (C) terminus. In brief, an Xbal-Swal fragment encompassing the chimeric Ad fiber gene was synthesized (Genscript; Piscataway, NJ) and used to replace the Xbal-Swal fragment of the wild-type Ad sequence within the pAdEasy-1 plasmid (Agilent Technologies; Santa Clara, CA), introducing the T4 phage rod-like trimeric fibritin molecule. Ampicillin-resistant colonies were selected following transformation. DNA was extracted, and identities of positive clones were confirmed by restriction digestion and PCR. A pIX-RFP reporter gene was introduced into the aAdEasy-1 by homologous recombination [17] with a modified pShuttle vector containing a wild-type Ad5 E1A gene and the mCherry coding sequence inserted downstream of the Ad5 minor capsid pIX gene to generate a C-terminal pIX fusion protein (pShuttle-E1A-pIX-RFP), a kind gift from Dr. Anton Borovjagin (University of Alabama at Birmingham, Birmingham, AL). Recombinants were selected on kanamycin agar plates and confirmed by restriction digestion and PCR. DNA sequencing was done to confirm the identity of the inserted fragments (Figures 1,2).

**Figure 1** A schematic representation of the chimeric fiber-fibritin-NK2 (FF-NK2) construct in the context of the Ad5 vector.

**Figure 2** A schematic representation of the interaction between the chimeric Ad5 fiber and c-Met.
the rescued virions were used to re-infect 293 cells at an optimal MOI. The recombinant Ad virions were purified by equilibrium ultracentrifugation on CsCl gradients. The virus titer of each Ad preparation was determined by spectrophotometry using a conversion factor of 1.1 × 10^13 viral particles per absorbance unit at 260 nm. The multiplicity of infection (MOI) was determined using an Adeno-X Rapid Titer Kit (Clontech; Mountain View, CA) and represents the number of infectious units (ifu) of the virus. Viruses were stored at -80°C until use.

Flow cytometry

A FITC-labeled anti-c-Met antibody and its isotype control (R&D Systems), a PE-labeled anti-hCAR antibody (Santa Cruz Biotechnology; Dallas, TX) and its isotype control (Abcam) were used for this assay. For each cell line, 1 × 10^5 cells were incubated with the antibodies for 45 min at 4°C in the dark. After incubation, the cells were washed twice with PBS, pelletted, resuspended in 400 µL PBS, and analyzed using a FACS Caliber cytometer (Becton Dickinson) using Cell Quest software.

Measuring Ad infectivity

To determine the efficiency of infectivity, CFPAC-1, PANC-1, AsPC-1 and BxPC-3 cells were seeded at 1 × 10^5 cells per well in 24-well tissue culture plates 1 day before infection. The cells were infected with increasing titers of unmodified and modified Ads expressing the green fluorescent protein (GFP) marker (Ad5-CMV-GFP-Luc), and the red fluorescent protein (RFP) marker (Ad5-wt-pIX-RFP, Ad5-pIX-RFP-FF-NK2) in medium containing 2% FBS. After infection for 2h, the medium was changed to 10% FBS. At 24 and 48 h after infection, the cells were harvested, the media was changed to PBS, and the inclusions were quantified by flow cytometry using a FACS Calibur cytometer (Becton Dickinson; Franklin Lakes, NJ).

Measuring cytotoxicity in vitro

The PANC-1, CFPAC-1, AsPC-1 and BxPC-3 cells were seeded into 96 well plates at approximately 3 × 10^3 cells per well in medium containing 10% FBS. After 16 h, the cells were infected with increasing titers of Ads (Ad5-wt-pIX-RFP, Ad5-CMV-GFP-Luc, or Ad5-pIX-RFP-FF-NK2) at 0.1, 1, 10, 100, and 1000 ifu/cell in tissue culture medium containing 2% FBS. The cells were incubated for 2h before the media was aspirated and replaced with tissue culture medium containing 10% FBS. After 5 days, the experiment was ended; the media was removed, and the cells were fixed with phosphate-buffered saline (PBS; Invitrogen). To quantify cell killing, the cells were fixed and stained with 0.1% crystal violet in 70% ethanol, followed by washing with tap water to remove excess dye. The plates were dried, the dye was dissolved in acidified ethanol (EtOH + 4% 1M HCL). Cell killing was determined by measuring absorbance at 595 nm using a SpectraMax 190 plate reader (Molecular Devices; Sunnyvale, CA).

Statistical analysis

All data are expressed as means ± SEM unless otherwise specified. Statistical analyses were performed using Student’s t-test (two-tailed). A P-value <0.05 was considered to be statistically significant and is marked by an asterisk.

RESULTS

First, to determine the surface expression of c-Met and hCAR expression in pancreatic cancer cell lines, flow cytometry was performed. As shown in Figure (3), three of the human pancreatic cancer cell lines (BxPC-3, CFPAC-1, and AsPC-1) showed high levels of c-Met expression while a fourth cell line (PANC-1) showed a low-to-moderate level of c-Met. In contrast, analysis of hCAR demonstrated that one cell line had an undetectable level (PANC-1), one cell line had a low level (BxPC-3), and two cell lines had a low-to-moderate level (AsPC-1 and CFPAC-1) (Figure 4). These results are summarized as the mean fluorescence intensity (MFI) of each peak in Table (1).

Adenoviruses containing the green fluorescent protein (GFP) or red fluorescent (RFP) markers were used to determine the efficiency of infection in the human pancreatic cell lines. All the Ad vectors had an identical backbone, the difference being the incorporation of alternative modifications in the Ad fiber region. To quantify the percent of GFP or RFP-positive cells, we performed flow cytometry analysis of infected cells. The results are shown in Figure (5A-D) indicate the fiber-modified Ad (Ad5-pIX-RFP-FF-NK2) was highly efficient in infecting PANC-1, CFPAC-1, AsPC-1, and BxPC-3 cells compared to an unmodified wild-type Ad (Ad5-wt-pIX-RFP) or non-replicating Ad5 (Ad5-CMV-GFP-Luc). We noted that infective efficiency was not dependent on the absolute level of c-Met; for instance, PANC-1 has a low/moderate level of c-Met, yet it is highly sensitive to infectivity by our novel adenoviral construct.

We next compared the cytotoxicity profile of oncolytic Ads in the pancreatic cancer cell lines (Figure 6A-D). The novel oncolytic adenoviral construct demonstrated highly effective oncolytic activity against all pancreatic cancer cell lines. The oncolytic activity

Figure 3 Analysis of c-Met receptor levels in different human pancreatic cancer cell lines. Cell surface expression analysis of c-Met was performed using PANC-1, CFPAC-1, AsPC-1, and BxPC-3 human pancreatic cancer cell lines. A FITC-conjugated monoclonal antibody specific for the human c-Met receptor was used. The cells were incubated with PBS alone (unstained), an isotype control antibody or the receptor-specific antibody in dark at 4°C for 45 min. Following incubation, the cells were washed, resuspended in 0.4 mL PBS, and analyzed by flow cytometry. In each experiment, 10,000 cells were analyzed for each sample. Shown are representative results of three independent experiments.
Figure 4 Analysis of hCAR receptor levels in different human cancer cell lines. Cell surface expression analysis of hCAR was performed using PANC-1, CFPAC-1, AsPC-1, and BxPC-3 human pancreatic cancer cell lines. A PE-conjugated monoclonal antibody specific for hCAR was used. The cells were incubated with PBS alone (unstained), an isotype control antibody or the receptor-specific antibody in dark at 4°C for 45 min. Following incubation, the cells were washed, re-suspended in 0.4 mL PBS, and analyzed by flow cytometry. In each experiment, 10,000 cells were analyzed for each sample. Shown are representative results of three independent experiments.

Figure 5 Determination of infection efficiency. Flow cytometric analysis of (A) PANC-1 (B) CFPAC-1, (C) AsPC-1, (D) BxPC-3 human pancreatic cancer cells infected with of Ad5-wt-pIX-RFP (●), Ad5-pIX-RFP-FF-NK2 (▼) or Ad5-CMV-GFP-Luc (▲) vectors expressing green fluorescent protein marker. An analysis was performed for expression of GFP or RFP at 48 h after infection with increasing doses from 0.1 ifu/cell to 1,000 ifu/cell. Values represent the mean ± SEM of 3 replicate measurements. The Ad5-pIX-RFP-FF-NK2 infected cells were compared pair wise with cells infected with Ad5-wt-pIX-RFP using a two-tailed Student’s t-test. The differences between the groups were considered statistically significant (*) if P < 0.05.

Figure 6 Oncolytic activity of an oncolytic Ad construct in pancreatic cancer cell lines. Oncolysis was evaluated by crystal violet staining in the pancreatic cancer cell lines (A) PANC-1, (B) CFPAC-1, (C) AsPC-1, (D) BxPC-3 after infection with increasing doses from 1 ifu./cell to 100 ifu/cell of Ad5-wt-pIX-RFP (●), Ad5-pIX-RFP-FF-NK2 (▼) or Ad5-CMV-GFP-Luc (▲) vectors as described in Material and Methods. Values represent the mean ± SEM of 3 replicate measurements. The Ad5-pIX-RFP-FF-NK2 infected cells were compared pair wise with cells infected with Ad5-wt-pIX-RFP using a two-tailed Student’s t-test. The differences between the groups were considered statistically significant (*) if P < 0.05.

Table 1 Summary of c-Met and hCAR surface expression in cancer cell line. Each cancer cell line was analyzed for the cell surface expression of c-Met and hCAR receptors. The data are represented as mean fluorescence intensity (MFI) of gated unstained cells and cells stained with receptor-specific antibodies or isotype control antibodies.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>c-Met levels (MFI)</th>
<th>hCAR levels (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstained Isotype</td>
<td>c-Met Unstained Isotype hCAR</td>
</tr>
<tr>
<td>AsPC-1</td>
<td>3.99 4.19</td>
<td>25.21 14.27 19.46 36.45</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>1.77 1.88</td>
<td>13.16 2.60 3.03 3.93</td>
</tr>
<tr>
<td>CFPAC-1</td>
<td>3.93 4.18</td>
<td>34.75 13.45 18.77 43.49</td>
</tr>
<tr>
<td>PANC-1</td>
<td>4.82 4.42</td>
<td>8.45 22.89 32.58 39.49</td>
</tr>
</tbody>
</table>

of the novel Ad5-pIX-RFP-FF-NK2 was superior to either the wild type Ad5-wt-pIX-RFP or non-replicating viruses Ad5-CMV-GFP-Luc. The degree of oncolysis did not appear to correspond to the level of c-Met surface expression. For instance, although PANC-1 had low to moderate expression of c-Met, its effectiveness in killing tumor cells is similar to cells that have a high c-Met expression (i.e., BxPC-3).

DISCUSSION

Pancreatic adenocarcinoma remains a recalcitrant disease. Today’s 5% five-year survival rate for all stages of the disease has not improved much compared to the 3% survival rate seen in the early 1970s [1]. Despite recent advances, the therapeutic armamentarium against pancreatic adenocarcinoma is somewhat limited. Whether patients receive chemotherapy and/or radiation
therapy before or after surgery, the outcome remains dismal. It is likely that the majority of pancreatic cancers are chemo and radioresistant. Significant impact on this disease will, therefore, require novel therapeutics. Virotherapy represents one such option [19-21]. Compared to traditional fields of cancer therapy, oncolytic virotherapy is a relatively young field. A number of attenuated viruses such as the Herpes simplex virus-1, Vaccinia virus, Influenza virus, Newcastle disease virus, Poliovirus, Reovirus, vesicular virus, and adenoviruses have been tested in the preclinical and clinical setting [20-22]. The attenuated adenovirus-based vector (i.e., conditionally replicating adenoviruses or CRAds) is a promising oncolytic virus that is commonly studied because of its low pathogenicity, effective oncolytic capability, and enhanced transfer of therapeutic genes [23-25]. We have previously demonstrated the efficacy of using a modified chimeric adenovirus to target pancreatic cancer cells that overexpress the chemokine receptor CXCR4 [15], a seven-transmembrane G-protein-coupled receptor that appears to play a pivotal role in the pathogenesis of cancers [15,26]. Because not all pancreatic cancers overexpress CXCR4, we sought to find another target, which in this case, is the c-Met.

We demonstrated that Ad5-plX-RFP-FF-NK2 was highly efficient at infecting the human pancreatic cancer cells when compared to controls. It is of interest that the efficiency of infectivity was not entirely dependent on the absolute level of c-Met. The PANC-1 has the lowest c-Met surface expression compared to the other pancreatic cancer cell lines, yet our novel adenoviral construct was highly efficient in infecting and lysing the cells. The reason behind this is not clear, but one of the possible explanations may involve binding of the Ad5-plX-RFP-FF-NK2 vector to secondary Ad receptors. After initial binding to hCAR, an RGD motif on the Ad penton capsid protein interacts with integrins on the cell surface to trigger internalization [27]. Alternatively, it may involve the shedding of the c-Met extracellular domain from tumors that have a high c-Met expression, thus leading to competitive binding of the Ad5-plX-RFP-FF-NK2, thereby reducing the amount of the novel constructs that are available for binding to tumor cells [28]. Selectivity of the oncolytic viruses towards pancreatic cancer cells is an important component of this novel construct. In this study, we have not tested the virus on c-Met negative cells. However, future experiments against HPDE6c7 immortalized pancreatic cancer cells, as well as other c-Met negative cell lines, will be performed. Additionally, we plan to design an siRNA against c-Met and then test it against our pancreatic cancer cell lines to determine the level of infectivity and oncolytic activity. This approach will further help answer whether our novel oncolytic virus is specific to cells that overexpress c-Met.

One of the concerns with oncolytic virotherapy is its potential effect on normal cells. The c-Met and HGF genes are required for normal mammalian development and expression of c-Met is found in many organs including the liver, pancreas, prostate, kidney, muscle and bone marrow [29]. The MET gene is highly expressed during embryonic development but becomes quiescent in the adult. It can be reactivated during wound healing and following organ damage or inappropriately activated in oncogenic development [29]. Although it is not known what impact our oncolytic viruses will have on normal cells that normally express c-Met, we postulate that it will be minimal; unlike pancreatic cancer cells, normal quiescent cells do not overexpress c-Met, and therefore may be minimally affected by the viruses. Finally, we are designing a second-generation virus with a deletion of 24 amino acid residues in E1A gene (Δ24) that will restrict replication to cancer cells.

Ad5 virus infectivity is a complex process, involving a two-step entry pathway, using hCAR and integrins αvβ3 and αvβ5. Creating a recombinant Ad5 fiber protein containing the HGF isoform NK2 may alter the binding specificity to c-Met in the context of the assembled virion. This alteration could be one possible explanation for Ad infectivity observed even in low c-Met-expressing cell lines such as PANC-1. In addition, the naturally occurring HGF/NK2 isoform (the targeting moiety in our Ad5 vector) binds weakly to a specific motif within heparan sulfate proteoglycans (HSPGs) via the N-terminal domain [30]. This binding interaction may add to the complexity of Ad5-plX-RFP-FF-NK2 virus interaction with the cells. Another potential explanation is that differences in integrin expression among the cell lines may play a role accounting for the differences in infectivity levels. It is possible that integrin expression on PANC-1 cells compensated for the low c-Met levels; this hypothesis is supported by data from Takayama et al. [31].

The next step in characterizing this novel virus will be to test anti-cancer activity in a preclinical rodent model, and eventually bring it into a clinical trial. The preferred route of delivery for any oncolytic viruses is intravenous, although there are hurdles that are needed to overcome before it can be widely applied in the clinical setting. Following intravenous injection, oncolytic viruses are neutralized by preexisting antibodies and complement activation, antiviral cytokines, tissue-resident macrophages, as well as sequestered into the reticuloendothelial system [32]. Because of these limitations, our future goal is to directly inject the oncolytic viruses into the tumor mass, either via CT-guided or ultrasound guided techniques. This route is especially ideal for patients with locally advanced, unresectable pancreatic cancer, whereby a conversion from an unresectable to a resectable cancer can be a novel alternative to conventional chemoradiation therapy.

In summary, we demonstrated through a step-wise approach that by replacing part of the fiber of the wild-type Ad5 with the fused T4-phage fibrinogen protein linked with the isoform NK2 (a competitive antagonist of the HGF/c-Met association), we were able to efficiently transduce and increase the oncolytic activity of the oncolytic Ads against an array of human pancreatic cancer cells. This novel recombinant oncolytic virus will be further investigated in vivo.

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REFERENCES


