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A targeting ligand enhances infectivity and cytotoxicity of an oncolytic adenovirus in human pancreatic cancer tissues

Yuki Yamamoto [a,c], Nobuyoshi Hiraoka [b], Naoko Goto [a], Yosei Rin [a,c], Kazuki Miura [a,c], Kenta Narumi [a], Hiroaki Uchida [c], Masatoshi Tagawa [d], Kazunori Aoki [a,*]

[a] Division of Gene and Immune Medicine, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
[b] Division of Molecular Pathology, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
[c] Laboratory of Oncology, Tokyo University of Pharmacy and Life Sciences, 432-1 Horinouchi, Hachioji, Tokyo 192-0355, Japan
[d] Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute, 666-2 Nitona-chou, Chuo-ku, Chiba 260-0801, Japan

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ABSTRACT

The addition of a targeting strategy is necessary to enhance oncolysis and secure safety of a conditionally replicative adenovirus (CRAd). We have constructed an adenovirus library displaying random peptides on the fiber, and have successfully identified a pancreatic cancer-targeting ligand (SYENFSA). Here, the usefulness of cancer-targeted CRAd for pancreatic cancer was examined as a preclinical study. First, we constructed a survivin promoter-regulated CRAd expressing enhanced green fluorescent protein gene (EGFP), which displayed the identified targeting ligand (AdSur-SYE). The AdSur-SYE resulted in higher gene transduction efficiency and oncolytic potency than the untargeted CRAd (AdSur) in several pancreatic cancer cell lines. An intratumoral injection of AdSur-SYE significantly suppressed the growth of subcutaneous tumors, in which AdSur-SYE effectively proliferated and spread. An ectopic infection in adjacent tissues and organs of intratumorally injected AdSur-SYE was decreased compared with AdSur. Then, to examine whether the targeting ligand actually enhanced the infectivity of CRAd in human pancreatic cancer tissues, tumor cells prepared from surgical specimens were infected with viruses. The AdSur-SYE increased gene transduction efficiency 6.4-fold higher than did AdSur in single cells derived from human pancreatic cancer, whereas the infectivity of both vectors was almost the same in the pancreas and other cancers. Immunostaining showed that most $EGFP^+$ cells were cytokeratin-positive in the sliced tissues, indicating that pancreatic cancer cells but not stromal cells were injected with AdSur-SYE. AdSur-SYE resulted in a stronger oncolysis in the primary pancreatic cancer cells co-cultured with mouse embryonic fibroblasts than AdSur did. CRAd in combination with a tumor-targeting ligand is promising as a next-generation of oncolytic virotherapy for pancreatic cancer.

1. Introduction

Pancreatic cancer is the leading cause of cancer death, and it is considered one of the most lethal cancers in Japan as well as in the Western countries [1–3]. Despite the recent advances in therapeutic and diagnostic modalities [4,5], overcoming it remains one of the most formidable challenges in oncology today. Complete surgical resection has traditionally been considered the only curative treatment, but high rates of local and systemic failure persist in patients who undergo curative resections, and a 5-year survival rate is less than 5% [1]. This high mortality is due to the high incidence of metastatic disease at the time of diagnosis, a fulminant clinical course and the lack of adequate systemic therapies. In the locally advanced cases also, the development of strategies to strongly control local lesions and prevent distant metastases is an important issue. In spite of the fact that many studies have been tried to improve the outcomes, a novel approach is needed [3,5].

The conditionally replicative adenoviruses (CRAds), which are genetically programmed to replicate within tumor cells but not in normal cells and directly induce cytotoxic effects via cell lysis, are currently being explored in preclinical and clinical studies of various cancers such as head and neck cancer, pancreatic cancer, ovarian cancer, prostate cancer and malignant glioma [6–8]. Progress in the clinical studies of CRAds for solid cancers has been clarifying the need for two issues to be addressed [6,7,9]. First, tumor oncolytic activity should be enhanced to achieve any significant antitumor response. Since, in general, the oncolytic activity of CRAds is closely related with the infectious ability to the targets, the improvement of viral infectivity to tumors is necessary. However, an undesirable viral spread to adjacent tissues should be strictly limited around the virus-replicating tumors to reduce any adverse effect. In addition, CRAds leak from the tumors into systemic

* Corresponding author. Tel.: +81 3 3542 2511(4401); fax: +81 3 3248 1631.
E-mail address: kaoki@ncc.go.jp (K. Aoki).

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circulation often causes ectopic infection to vital organs such as the liver [10]. Therefore, suppression of naïve tropism for the reduction of unnecessary infection in non-target tissues and organs, and the reinforcement of a tumor-targeting potential in combination with the reduced tropism should solve the issues of conventional CRAds therapy [10,11].

Most of the presently used CRAds are based on serotype 5 (Ad5), whose entry into susceptible cells requires two distinct and sequential steps. The initial step is facilitated by an interaction of the fiber protein with its cellular receptor coxsackievirus and adenovirus receptor (CAR) [12]. Following attachment to CAR, internalization of the virus is promoted by the interaction of the penton base with αv integrins on the cell surface [12]. Retargeting has been achieved by direct genetic modifications of the capsid proteins: targeting ligands can be incorporated into the C-terminal and HI-loop of fiber proteins ablated for native tropism through the loss of binding with CAR and/or αv integrins [6,12]. However, the redirection of adenovirus vectors by engineering the capsid-coding region has shown limited success because proper targeting ligands are generally unknown. To overcome this limitation, we have developed a system for producing adenoviral libraries displaying a variety of peptides on the HI-loop of the fiber knob, and its screening has led to successful selections of several particular adenoviral vectors with high infectivity in target cells [13–17]. SYENFSA (SYE) is one of the selected sequences from the library by screening on the AsPC-1 pancreatic cancer cell line *in vitro*, and the adenovirus displaying the sequence showed higher infectivity in four of five pancreatic cancer cell lines [15].

To date, 2 types of CRAds have been developed. Type I is for introducing a mutation in the E1 region, and the function of these missing genes may be complemented by genetic mutation in tumor cells such as p53 mutation. Type II is for constructing viruses in which the transcription of E1 genes is restricted to tumor cells by either a tumor or tissue-specific promoter [6–8]. Survivin (Sur) is an anti-apoptotic protein involved in mitotic regulation during embryotic and fetal development, but its expression is generally undetectable in terminally differentiated adult tissue [18]. The promoter activity, largely silent in normal cells, is prominently expressed in tumor tissues including pancreatic cancer, and cells transfected with a reporter gene under the control of a Sur promoter exhibit cancer-specific activity *in vitro* and *in vivo* [19,20]. Therefore, CRAds, in which the transcription of E1 genes is restricted to tumor cells by the Sur promoter, were widely studied [21]. In this study, we constructed a Sur promoter-regulated CRAd displaying the pancreatic cancer-targeting sequence SYE (AdSur-SYE) to enhance oncolysis and secure safety. The therapeutic usefulness of AdSur-SYE was validated in surgical specimens of human pancreatic cancer as a preclinical study.

2. Materials and methods

2.1. Cell lines

Used in this study were a human embryonic kidney cell line (293), human pancreatic cancer cell lines (AsPC-1, BxPC-3, Panc-1 and MIAPaCa-2), a human prostate cancer cell line (PC3), a human dermal fibroblast and a mouse embryonic fibroblast (MEF). All the cancer cell lines were obtained from the American Tissue Culture Collection (ATCC; Rockville, MD, USA). Fibroblast was purchased from PromoCell GmbH (Heidelberg, Germany) and MEF was from ReproCell (Kanagawa, Japan). 293 cells, MIAPaCa-2 cells and MEFs were cultured in Dulbecco's modified eagle's medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) with 10% fetal bovine serum (FBS); pancreatic cancer cell lines except for MIAPaCa-2 and PC3 were cultured in an RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) with 10% FBS. Fibroblasts were cultured in the fibroblast growth medium (PromoCell GmbH). The 293–38 is a high-efficiency virus-producing clone of 293 cells [13].

2.2. Human surgical specimens

Surgical specimens (15 pancreatic ductal adenocarcinomas, 1 intraductal papillary mucinous neoplasm, 1 metastasis of renal cancer, 1 gallbladder cancer, 1 duodenal cancer, 6 pancreases and 1 liver) were obtained according to the Declaration of Helsinki Principles and the guideline of the Ethics Committee of the National Cancer Center (Tokyo, Japan). None of the patients had received any prior therapy. Tissues were processed into single cells or small pieces (1–4 mm in diameter), and were cultured in RPMI-1640 medium with 10% FBS.

2.3. Plasmids and recombinant adenovirus vectors

The 0.5-Kb survivin regulatory region [20] was inserted into the pGL3 basic vector (Promega, Madison, WI, USA) that contains the firefly luciferase gene, and designated as a pSur-Luc. A pRL-SV40 plasmid (Promega) expresses *Renilla* luciferase gene under the control of a SV40 promoter.

Adenovirus vectors were constructed as previously described [14,15,22]. The adenovirus vectors except for Ad-EGFP and AdΔE1-AP include 4 point mutations in the AB-loop of the fiber knob that reduces CAR binding. Ad-EGFP and AdΔE1-AP have a wild type of fiber. The AdSur-SYE and AdSur contain a 0.5-Kb survivin regulatory region upstream of the adenoviral E1 gene [20]. The AdΔCAR-SYE, AdΔCAR and Ad-EGFP contain a wild type of E1 region. The AdΔCAR-SYE, AdSur-SYE and AdLucEGFP-SYE have a SYE sequence in the HI-loop on the fiber knob [15,16]. The virus vectors contain a CMV promoter, the enhanced green fluorescent protein (EGFP) gene and a SV40 poly(A) signal in place of the E3 region, except for the AdΔE1-AP, AdLucEGFP and AdLucEGFP-SYE. In these viruses, the E1 gene is replaced with the CMV promoter-driven alkaline phosphatase or luciferase-EGFP fusion gene (LucEGFP), respectively (Fig. 1). The adenovirus vectors were expanded in the 293–38 cells. Physical particle concentration (viral particles (vp)/ml) of virus preparation was determined by optical absorbance (OD_{260}) [23].

2.4. Assay for luciferase activity

The cells were seeded at 1×10^5 per well in 24-well plates and transfected with both of pSur-Luc and pRL-SV40 by the lipofection method (Lipofectamine 2000 Reagent; Life Technologies Corp., Carlsbad, CA, USA). The cells were harvested with 100 μl of reporter lysis buffer (Promega) 48 h after the transfection. The light units of firefly and *Renilla* luciferase activities were assessed by dual luciferase reporter assay (Promega) using a luminometer (MiniLumat LB9506; EG&G Gerthold, Vilvoorde, Belgium). The sliced tissues prepared from surgical specimens of human pancreatic cancer (~1 mm in diameter) were infected with 3×10^9 vp of AdLucEGFP-SYE and AdLucEGFP. Twenty-four hours after the infection, the tissues were lysed with an equal volume of reporter lysis buffer. Twenty microliters of cell lysates were mixed with 100 μl of luciferase assay substrate (PicaGene; Toyo Ink CO., LTD., Tokyo, Japan). The light units of luciferase activity were measured using a luminometer (MiniLumat LB9506).

2.5. In vitro cell growth assay

The cells were seeded at 3×10^3 per well in 96-well plates and infected with viruses at 300, 1×10^3, 3×10^3, 1×10^4 and 3×10^4 vp/cell. The 1×10^4 of single cells prepared from surgical specimens of pancreatic cancers were co-cultured with 1×10^4 of MEF as feeder cells per well in 96-well plates and were infected with viruses at 1×10^3, 3×10^3, 1×10^4 and 3×10^4 vp/cell. The cell numbers were assessed by a colorimetric cell viability assay using a water-soluble tetrazolium salt (Tetracolor One; Seikagaku Corp., Tokyo, Japan). The absorbance was determined by spectrophotometry using a wavelength of 450 nm with 600 nm as a reference.