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Construction of conditionally replicating adenovirus expressing staphylococcal enterotoxin A gene: potential usefulness for anti-tumor therapies

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Abstract. – OBJECTIVE: The aim of this study was to construct a conditionally replicating adenovirus pPE3-SEA expressing staphylococcal enterotoxin A (SEA) gene.

MATERIALS AND METHODS: A full-length SEA gene fragment was cloned into pENTR12 plasmid to obtain a recombinant viral plasmid pENTR12-SEA. The pENTR12-SEA plasmid was co-transfected into HEK293 cells along with pPE3-ccdB, which encoded for the virus backbone, to generate recombinant adenovirus pPE3-SEA vector. Amplified pPE3-SEA vectors were purified, and viral titer was determined using the 50% tissue culture infective dose method.

RESULTS: The PCR, restriction enzyme digestion, and sequence analyses proved successful construction of replicating oncolytic adenovirus pENTR12-SEA and recombinant SEA expressing oncolytic adenovirus pPE3-SEA. The viral titer was 2.5×10^{10} pfu/ml.

CONCLUSIONS: We successfully constructed conditionally replicating adenovirus pPE3-SEA which can be utilized for experimental studies of tumor-targeted therapies.

Key words:
Gene, Oncolytic viruses, Adenoviridae, Enterotoxins, Neoplasms.

Introduction

Malignant diseases are a substantial threat to human health and burden to health care system. The current cytotoxic agents exert cytotoxic adverse effects on the body. Therefore, a development of novel agents that would specifically target tumor cells is the focus of current anti-cancer studies. In our study, we generated conditionally replicating oncolytic adenovirus (Ad) by genetically engineering Ad to yield tumor-killing abilities in order to make it usable as a tumor-targeting carrier of antitumor agents.

Bacterial superantigens (SAg) are potent T cell activators and include staphylococcal enterotoxin (SE) SEA, SEB, SEC1, SEC2, SED, and SEE, and toxic shock syndrome toxin 1 (TSST1). SEA is the most studied SAg. SEA activates T cells by direct binding to major histocompatibility complex class II molecules on antigen presenting cells and T-cell receptors of a subset of T cells-bearing particular Vb-chains. Targeting the superantigens in tumors induces a strong, local cytotoxic T-cell attack that selectively kills tumor cells[1]. A non-replicating oncolytic Ad vector, pDC318-SEA, carrying the SEA gene was successfully constructed in our previous study[2]. In the present study, a conditionally replicating oncolytic Ad pENTR12-SEA carrying SEA gene was genetically engineered to further study the tumor-killing effect of this replicating oncolytic virus.

Materials and Methods

Reagents

Viral vectors, including pENTR12-Linker and pPE3-ccdB, and HEK293 cells were purchased

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from the Virus and Gene Therapy Center of Eastern Hepatobiliary Surgery Hospital of Shanghai Second Military Medical University (Shanghai, China). Gel extraction kit, PCR product purification kit, plasmid DNA purification kit, Dulbecco's Modified Eagle Medium (DMEM) and viral DNA isolation kit were from Qiagen (Nanjing, China). Lipofectamine 2000 was purchased from Gibco BRL (Grand Island, NY, USA). DNA ligation solution I and Taq DNA ligase were obtained from TakaRa (Tokyo, Japan).

Isolation of SEA gene

The genetically engineered SEA expressing oncolytic Ad vectors were digested using restriction enzymes Spel I and Sal I. The digestion was performed in the system that comprised 3.0 μl of pDC318-SEA vector, 3.0 μl of Spe I, 7.0 μl of 10X BSA, 7.0 μl of buffer, and H_2O for a total volume of 70 μl. After incubation of sample in water bath for 6 hours at 37º C, Sal I (3.0 μl), 10X BSA (3.0 μl), buffer (10 μl), and H_2O (14 μl) were added to the sample, making the total volume of 100 μl. After another 8 hours of incubation in water bath at 37º C, samples were separated on 1.2% agarose gel. The 774 bp fragment of SEA gene was isolated and purified using QIAquick Gel Extraction kit from Qiagen (Nanjing, China).

Construction of conditionally replicating oncolytic Ad vector pENTR12-SEA

The Spe I/Sal I digested SEA gene fragment was ligated with pENTR12 digested with Spe I/Sal I. The ligation reaction was performed at 12º C for 12 hours in a 20-μl solution containing pDC318-SEA/Spe I Sal I (2 μl), pENTR12-Liner/Spe I Sal I (8 μl), and Solution I (10 μl). The ligated product was transformed into competent *E. coli* DH5α cells. The transformed culture was plated on agar plates containing kanamycin. After incubation for 10-12 hours at 37º C, a single colony of *Escherichia coli* (*E. coli*) cells was isolated and propagated for 12 hours with shaking at 37º C in LB solution containing kanamycin. Then, plasmid DNA was extracted from positive clones. The identity of recombinant plasmids was confirmed by digestion with restriction enzymes. Successfully engineered plasmid was named "pENTR12-SEA".

Recombination and identification of SEA expressing conditionally replicating oncolytic Ad vector

The pENTR12-SEA recombinant plasmid and the plasmid containing viral structural genes, pPE3-ccdB, were co-transfected into HEK293 cells using lipotransfection. After 9-14 days, the co-transfected viral plaques were isolated and purified 3 times. The adenovirus DNA was extracted using QIAampDNA Blood Mini Kit from Qiagen (Nanjing, China) and verified by PCR. The resulting recombinant DNA was called "pPE3-SEA".

Amplification and titer analysis of SEA expressing viral vector pPE3-SEA

HEK293 cells were grown to 80-90% confluence in a 75-cm^2 flask in 20 ml of DMEM (10% fetal bovine serum, FBS). The medium was changed afterwards to 15 ml of DMEM (2% FBS), and 0.5 μl of initially amplified virus (i.e., amplified in a 24-well culture plate) was then inoculated in the HEK293 cell solution, slowly shaken 3 times, and incubated in the incubator at 5% CO_2/37º C. After 48 hours of incubation, both supernatant and cell pellets were collected. After suspension in AD buffer, cell pellets were freeze-thawed 3 times (-80º C-37º C). The supernatant was collected after a 20-min centrifugation at 600 g. This supernatant was mixed with previously collected viral supernatant. The combined supernatant was then freeze-thawed 3 times as before. New supernatant was obtained by centrifugation as above. The virus in the supernatant was repeatedly amplified using the above procedures until the required amount was obtained, and the viral titer was determined using the 50% Tissue Culture Infective Dose ($TCID_{50}$) method.

Purification of virus plaque

The 293 cells were seeded on a 24-well plate before infection with the recombinant virus of different dilutions for 4 hours. The cells were supplemented with DMEM (5% FBS) and low melting gel, as described below. After 4-5 days, appropriate dilutions were picked up, and any well-separated virus plaques were subsequently subjected to PCR or plaque hybridization assays.

Virus preparation and CsCl gradient purification:

The 293 Cells were seeded onto 30-40 10-cm dishes and inoculated with 10 μl of virus (approximate 10^7-10^8 pfu/ml). After infection for 4-7 days, 500 μl of 10% NP 40 were added to each dish to lyse the cells. Whole cell lysates were collected and centrifuged at 12,000 rpm for 10 min. Cell debris were discarded and supernatants collected. To each 100 μl of supernatants, 50 μl of PEG8000

(20% PEG8000, 2.5 M NaCl) were added, and the mixture was kept on ice for one hour to precipitate virus, followed by centrifugation at 12,000 rpm for 20 min. The supernatant was discarded and the precipitate was suspended in a CsCl solution 10 mL with the density of 1.10 g/mL (in 20 mM Tris-HCl, pH 8.0), followed by centrifugation at 4° C/7,000 rpm for 5 min. Then, virus suspension was collected. Preparation of CsCl gradient was done as follows: 2.0 ml of CsCl (with the density of 1.40 g/ml, the same solvent was used) was added gently via a Pasteur pipette, followed by the addition of CsCl solution (3.0 ml) with the density of 1.30 g/mL, and virus suspension (5 ml). The mixture was centrifuged at 20,000 rpm for 2 hours. Virus straps with the density of 1.30-1.40 g/ml were collected and transferred to a dialysis bag, which was boiled with 10 mM EDTA Na_2 for 10 min before use. The straps were dialyzed in a dialysis buffer, which consisted of sucrose (50 g) dissolved in 1 M Tris-HCl solution (10 ml, pH 8.0) and 1 M $MgCl_2$ (2 ml), and water to 1000 ml, at 4° C overnight, with a change of dialysis buffer during this process. The virus was collected for titer determination. The CsCl solution of three different densities (dissolved in sterile 20 mM Tris, pH 8.0) were prepared and preserved at 4° C.

Virus titer

Determination of Plaque Forming Unit (pfu) was done as follows. The 293 cells were seeded onto 60-mm dishes and grown for 24 hours. After administration of virus of different dilutions and subsequent infection at 37°C for 2 hours, low melting gel (5% FBS, 1.25% Agarose) was added. The pfu was determined at approximate day 9. In the above procedures, virus plaque was picked up from the culture at the lowest concentration and was used for virus purification.

Determination of Optical Particle Unit (OPU): after CsCl centrifugation and purification, virus particles were diluted to yield OD values at 260 nm in the range of 0.1-1.0. The OPU of virus was estimated using the following formula: OPU/ml = OD_{260} × dilution factor × 1.1 × 1012. The correlation between PFU and OPU for appropriately purified virus was approximately PFU/ml = OPU/50.

Results

Isolation and identification of SEA gene fragment

The pDC318-SEA and pENTR12 plasmids were digested with Spe I/Sal I, and digested products were separated on 1.2% agarose gel. The separated gene fragments were extracted using gel extraction kit. The 771 bp SEA gene fragment and 3031 bp pENTR12 plasmid are shown in Figure 1.

Identification of SEA expressing conditionally replicating oncolytic Ad vector – pENTR12-SEA

The pENTR12-SEA vector and pENTR12 plasmid were digested and processed as above.

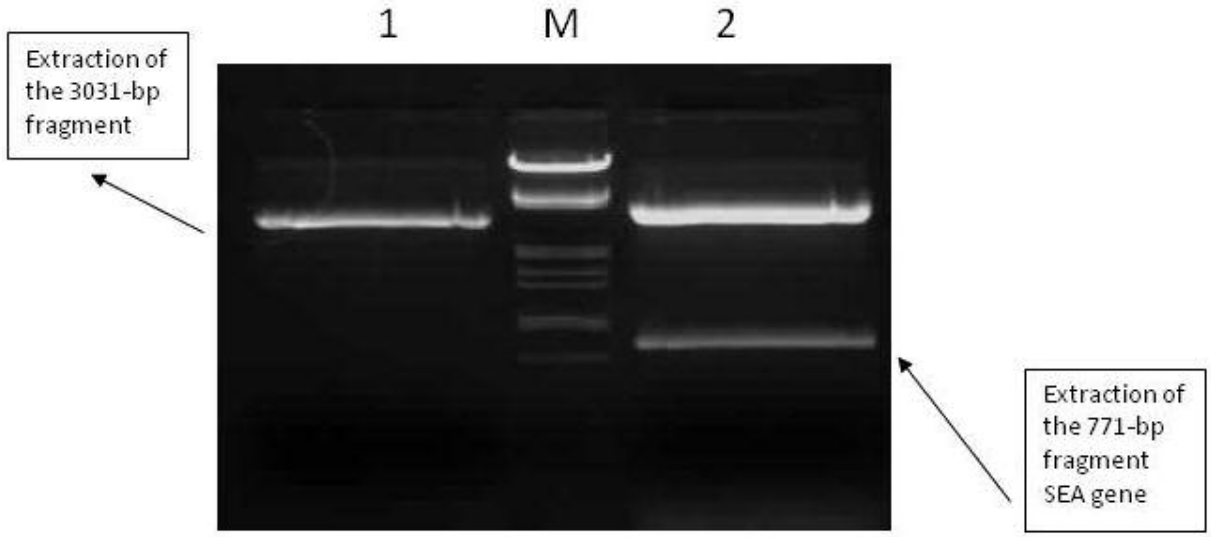

Figure 1. Restriction enzyme digestion of pDC318-SEA and pENTR12. M: DNA markers; 1: pENTR12 plasmid; 2: SEA gene and pDC318 plasmid.

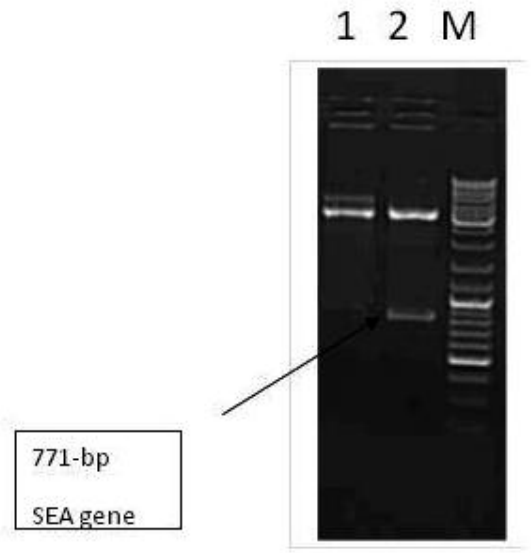

Figure 2. Restriction enzyme digestion of pENTR12-SEA and pENTR12 plasmids. M: DNA markers; 1: pENTR12 plasmid; 2: SEA gene and pENTR12 plasmid.

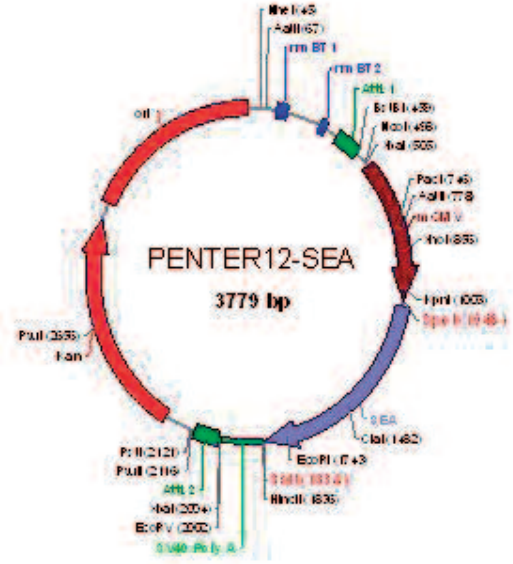

Figure 3. Plasmid structure of SEA expressing conditionally replicating oncolytic adenovirus vector pENTR12-SEA.

The 771 bp SEA fragment is shown in Figure 2. The structure of pENTR12-SEA is demonstrated in Figure 3.

Identification of SEA expressing conditionally replicating oncolytic Ad viral vector pPE3-SEA

The vector pPE3-SEA was digested with Xho I and separated using 1% agarose gel electrophoresis. Eight fragments (4908, 2466, 1445, 595, 14500, 4995, 498, and 8622 bp) were identified by gel electrophoresis. Thereby, the construction of the recombinant vector was confirmed (Figures 4 and 5).

Analysis of viral titer

The pPE3-SEA was amplified to yield more than 2 x 10^{10} pfu/ml, and purified and concentrated by centrifugation. The viral titer was determined to be at 2.5 × 10^{10} pfu/ml.

Discussion

Superantigens (SAg) are a class of bacterial and viral proteins that systemically activate T cells and macrophages. Endogenous viral SAg, similar to mouse mammary tumor virus SAg or exogenous bacterial SAg, belong to this class. Utilizing the immunostimulatory effect of SAg, extensive studies have been conducted on the application of SAg in anti-tumor therapy. Currently, most effective SAg is 5T4Fab-SEA tumor-targeting SAg that has undergone a phase III clinical trial in advanced renal carcinoma[3]. SEA has been evaluated for its T-cell stimulating effect, cytotoxic lymphocyte proliferation, and antitumor efficacy in our previous study[4]. Our previous study demonstrated that SEA activates cytotoxic lymphocytes and exhibits powerful *in vitro* tumor-

Figure 4. Gel electrophoresis of pPE3-SEA digested with Xho I restriction enzyme. M: DNA markers; 1: the Xho I digested fragments of pPE3-SEA. Eight DNA fragments were observed.

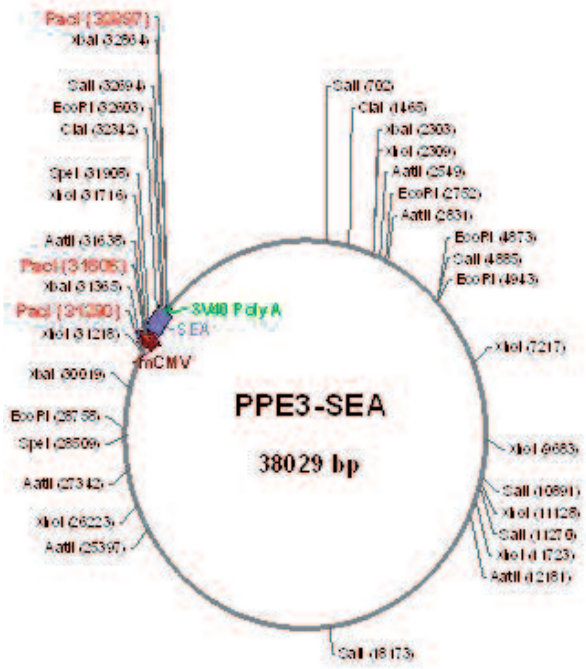

Figure 5. Structure of SEA expressing conditionally replicating oncolytic adenovirus pPE3-SEA.

killing effects on E-J bladder carcinoma cells[5]. Further, animal studies showed that SEA inhibits tumor growth *in vivo* with a tumor inhibitory rate of 84%[5]. The combined application of SEA and dendritic cells led to highly potent and specific anti-tumor effect on bladder carcinoma, improving the antigen-presenting effect of dendritic cells in tumors[6].

Although SAg are promising biological agents against tumors, many limitations exist for their application. The disadvantage of the treatment by using only SAg is that cytotoxicity mediated by SAg predominantly kills positive tumor cells of MHC-II and is less efficient in in killing negative tumor cells. Since positive rates of MHC-II in tumor cells is generally very low, and there is an obvious heterogeneity of expression, the overall efficiency of separate application of SAg is far from ideal, and is associated with toxic side effects due to effects on positive non-tumor cells of MHC-II. Further, T cells activated by fusion protein of monoclonal antibody are of SEA reactivity rather than tumor specific T cells. In addition, not all bladder cancers express BDI-1 and other relevant bladder tumor antigens, therefore, SAg of fusion protein of monoclonal antibody still lacks anti-tumor broad-spectrum applicability. The anti-tumor immune response induced by it is not very satisfactory. Therefore, finding an efficient and suitable carrier to deliver therapeutic agents to the tumor is a critical step toward a more effective tumor-targeting therapy.

Oncolytic viruses are a type of viruses capable of specifically infecting and lysing cancer cells while leaving healthy cells unaffected[7]. As early as 100 years ago, researchers found that spontaneous remission occurrs in some cancer patients who underwent virus infection. This led to studies of oncolytic viruses at the early 20th century[8]. Since mid 1990s, with the rapid development of molecular biology, molecular abnormalities of tumor cells have been gradually revealed. Further, a better understanding of the viral gene structure, as well as of the mechanism of the virus-tumor cell and virus-immune system interactions, facilitated the application of oncolytic viruses in anti-tumor therapy. Currently, a wide range of oncolytic virus has been constructed and applied in the treatment of cancer, including adenovirus, herpes simplex virus, Newcastle disease virus, reovirus and other viruses. More intensive research has been conducted on the gene structure and regulation of expression of adenoviruses. An example is Onxy-015 (dl152) which is the earliest reported oncolytic virus[9]. The Onxy-015 (dl152) became the first oncolytic virus to be used in cancer patients in solid tumor and is currently undergoing a phase III clinical trial[10].

Oncolytic adenovirus is constructed through modification of the genes E1A and E1B which are essential for replication but not essential for infecting tumor cells. This produces a replication-deficient oncolytic adenovirus with tumor-specific attacking capabilities[11]. Oncolytic adenovirus has emerged as a new therapy for the treatment of cancer[12]. In addition, carrying exogenous therapeutic genes can significantly increase the tumor-killing effect of replicating adenovirus. In our study, the SEA SAg was cloned into the genes of conditionally replicating oncolytic adenovirus. Further recombination and assembly generated a bio-active SEA expressing conditional oncolytic adenovirus. The advantages of this oncolytic adenovirus are as following: (1) By using superantigen SEA gene and with the local high efficient expression of SAg SEA gene in the tumor, strong anti-tumor immune response is stimulated, which remarkably improves the tumor-damaging effect; (2) oncolytic adenovirus is a vector that can only replicate in tumor cells; (3) virus replication in tumor cells can lyse tumor cells by itself.

The therapeutic use of oncolytic viruses needs to be carefully considered. Bladder is a cavernous organ which is communicated with exterior, serving as a "meaty test tube". The anatomical and histopathological feature like this enables the Sag-adenovirus construct to reach the bladder during bladder irrigation, thus limiting the effect of the construct only to the tumor-affected area. After being perfused into the bladder, the Sag-adenovirus construct markedly enhances partial immunologic function. SAg will get into direct contact with the bladder tumor cells and activate cytotoxic cells. Perfusing of oncolytic virus into the bladder can effectively infect the bladder cancer cells and obtain an effective expression, thus improving the killing effect for tumor cells and minimize the infection of the normal tissue cells. In addition, bladder irrigation is a simple procedure in operation and can be repeated multiple times, thus greatly enhancing the anti-tumor effect.

Conclusions

Using the SAg SEA as a target gene and recombinant adenovirus as a vector, we have constructed the oncolytic adenovirus with SEA gene and obtained higher virus titer. This construct will be useful for future studies on tumor-targeted gene therapies.

Conflict of interest

The Authors declare that they have no conflict of interests.

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