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Adenovirus-mediated expression of BmK CT suppresses growth and invasion of rat C6 glioma cells

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Received: 17 December 2012 / Accepted: 11 February 2013 / Published online: 27 February 2013
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Abstract  BmK CT, one of the key toxins in the venom of the scorpion, Buthus martensi Karsch, can interact specifically with glioma cells as a chloride channel blocker and inhibit the invasion and migration of those cells via MMP-2. A recombinant adenovirus, Ad-BmK CT, was constructed and characterized by in vitro and in vivo studies, using MTT cytotoxicity assay and the glioma C6/RFP (red fluorescence protein)/BALB/c allogeneic athymic nude mice model, respectively. The adenovirus-mediated expression of BmK CT displayed a high activity in suppressing rat C6 glioma cells growth and invasion thereby suggesting that this recombinant adenovirus may be a powerful method for treating glioblastoma.

Keywords  Adenovirus · BmK CT · Gliomas · Scorpion toxin

Introduction

Malignant gliomas, generated by the transformation of astrocytes, oligodendrocytes or their progenitor cells, are usually resistant to available therapies since they fail to undergo apoptosis upon anticancer treatment (Wang and Ji 2005; Sontheimer 2003). In the last 20 years, standard treatments (a combination of surgery, radiotherapy and chemotherapy) have not produced a significant improvement in the survival of glioma patients (Shen et al. 2005). Gene therapy is proposed as a novel therapeutic strategy for glioma. Adenovirus-mediated delivery of the conditional cytotoxic gene has been proposed as an adjuvant gene therapy approach for the treatment of glioma (King et al. 2008).

Chlorotoxin (Cltx) is one of the key toxins in the venom of the scorpion, Leiurus quinquestriatus, and it can bind specifically to glioma cell surface as a specific chloride channel blocker (Soroceanu et al. 1998; Olsen et al. 2003; Veiseh et al. 2007). Results from matrigel invasion assay demonstrate that Cltx can inhibit the enzymatic activity of MMP-2, which is involved in cell migration and specifically up-regulated in gliomas (Deshane et al. 2003). Since Cltx could specifically bind to glioma cells, it is believed that intensive and localized radiation will be delivered to individually infiltrated tumor cells beyond the tumor margin while leaving surrounding normal tissue cells untouched. Targeted radiotherapy using 131I-chlorotoxin showed that it had a high concentration in tumors and low
concentration in normal organs in athymic nude mice bearing D54 MG cells xenografts in brain (Shen et al. 2005). A chlorotoxin-like peptide gene, BmK CT, was cloned and sequenced from the venom of Buthus martensi Karsch by Wu et al. (2000) and Zeng et al. (2000). Similarly, the significance of BmK CT has been well documented as a novel blocker of chloride channels and MMP-2 (Fu et al. 2007, 2011).

In this study, the recombinant adenoviral system was produced by a double-recombination event between co-transformed adenoviral backbone plasmid vector, pAdEasy-1, and a shuttle vector pShuttle-IRES-hrGFP-2 carrying the BmK CT gene. We have developed this delivery system that specifically targets BmK CT to glioma cells. The delivered BmK CT will then interact with the MMP-2 and/or pro-MMP-2 in the glioma cells, which may avoid immunologic rejection and degradation of BmK CT protein. Here, the potential therapeutic effects of Ad-BmK CT against rat glioma C6 cells are assessed in vitro and in vivo.

Materials and methods

Materials

The gene sequence of BmK CT was designed and synthesized by Sangon Biological Engineering Technology & Services Co, Ltd (Shanghai, China) according to the codon optimization in mammals. The plasmid pShuttle-IRES-hrGFP-2, pAdEasy-1, wild type adenovirus, human embryonic kidney cell line–AD293 and the rat glioma cell line–C6 were kindly provided by Prof. Jianing Wang (Institute of Clinical Medicine, People’s Hospital, Yunyang Medical College, Shiyan, Hubei, China). C6/RFP cells were C6 cells co-transfected with plasmids expressing the Red Fluorescence Protein gene and neomycin resistance gene. Lipofectamine 2000 was purchased from Invitrogen. The restriction endonucleases, Pme I and Pac I, were purchased from New England BioLabs (NEB) (Beijing, China). The BALB/c nude mice were purchased from Vital River Laboratories (Beijing, China). Dulbecco’s Modified Eagle Medium (DMEM) was from Gibco BRL, calf serum was from Hangzhou Evergreen Corp. All other reagents were of highest grades.

Cell culture

AD293 cells were cultured in DMEM medium supplemented with 15 % (v/v) heat-inactivated fetal bovine serum (FBS) and the rat glioma C6 cells were cultured in DMEM with 10 % (v/v) heat-inactivated FBS at 37 °C with 95 % air, 5 % CO₂ and 100 % relative humidity. C6/RFP cells were cultured in DMEM with 10 % (v/v) FBS and 4 mg G418/ml.

Construction of recombinant adenovirus Ad-BmK CT

PCR was employed to amplify the BmK CT gene from pUC-57-BmK CT using specific primers (Forward primer: 5’-TT GAT ATC ATG TGC GGC CCC TGC TTC-3’; Reverse primer: 5’-AT CTC GAG TCA GAT ACG GTT GCA CAG GC-3’). The forward and reverse primer contained EcoRV and XhoI restriction sites, respectively, which facilitated the cloning of the gene to the transfer vector pShuttle-IRES-hrGFP-2. The purified PCR product was subcloned into pShuttle-IRES-hrGFP-2.

The recombinant plasmid pShuttle-IRES-hrGFP-2-BmK CT was linearized with Pme I and then dephosphorylated with alkaline phosphatase (CIAP). The linearized pShuttle-IRES-hrGFP-2-BmK CT was transformed into the competent BJ5183 strain transformed with pAdEasy-1. The identification of recombinant adenoviral plasmid pAdEasy-BmK CT was performed by PCR.

pAdEasy-BmK CT was digested by Pac I and was lysed by 40 µl sterile water. Transfection of pAdEasy-BmK CT into AD293 cells was performed with Lipofectamine 2000 according to manufacturer’s instructions. The progress of the transfection was monitored by fluorescence microscopy.

The VP (viral particles) protocol was used to monitor the absorbance of disrupted virions at 260 nm as described by the Adenovirus Technologies (Application manual of Obiogene Version 1.4).

Infection assay

To detect the best multiplicity of infection (vp cell⁻¹) for adenovirus-mediated gene transfer, C6 cells were exposed to Ad-BmK CT or control (Ad) from 5 × 10³ to 5 × 10⁴ vp cell⁻¹ for 48 h. Fluorescence microscopy was used to observe cell viability according to
cell morphology. FACS was used to quantify the infection efficiency of the adopted vp cell$^{-1}$ according to the expression of GFP.

**Cytotoxicity assay**

C6 glioma cells were used for examining the effects of Ad-$BmK$ CT or Ad on cell growth by MTT assay. Equivalent numbers of C6 cells (≈10$^4$ cells well$^{-1}$) were plated in 96-well microtiter plates in 200 μl. After 12 h, the culture medium was removed and the cells were treated with the fresh medium containing Ad-$BmK$ CT and Ad, respectively. After treatment, cell culture medium in each well was replaced with 200 μl medium containing 0.5 mg MTT ml$^{-1}$, followed by incubation at 37 °C for 3 h. The medium was then carefully removed and 200 μl DMSO was added to each well. After gentle shaking at room temperature for 10 min, the absorbance was read at 490 nm.

**RT-PCR analysis of the expression level of $BmK$ CT**

To determine the expression level of $BmK$ CT in Ad-$BmK$ CT transfected AD293 and C6 cells respectively, the total RNA of cells were isolated using

![Fig. 1](image-url) Construction of recombinant Ad-$BmK$ CT. 
**a** The digestion product of recombinant plasmid pAd-$BmK$ CT by Pac I. Left lane, λDNA/HindIII Marker; right lane, pAdEasy-$BmK$ CT was digested by Pac I. 
**b** PCR analysis. Lane 1 and 2, PCR product positive control of $BmK$ CT; lane 3, PCR product of $BmK$ CT from pAdEasy-I template; lane 4, negative control; lane 5, PCR product of $BmK$ CT from pShuttle-IRES-hrGFP-2-$BmK$ CT vector template. 
**c** The progress of the transfection was monitored by fluorescence microscopy.
RNAisoPlus (TaKaRa). The first cDNA synthesis was performed as described by PrimeScript reagent kit, Perfect Real Time (TaKaRa). Primers used in the PCR were: human-actin-F (5'-ATCGTCACCAACTGGGCACGACATGG-3'); human-actin-R (5'-AGGCCAGCTCGTAGCTTCTTCCC-3'); rat-actin-F (5'-TCAGGTCATCATAGTGCAAT-3'); rat-actin-R (5'-AAAGAAAGGGTGTAAAACGCA-3'); BmK CT-F (5'-TGATATCATGTGCGCCCCTGTTTC-3') and BmK CT-R (5'-ATCTCGAGTCAGATACGGTTGCACAGGC-3').

Potential therapeutic assay of Ad-BmK CT against rat glioma C6 cells in vivo

Six-week-old female BALB/c allogeneic athymic nude mice were subcutaneously inoculated on their armpits of right anterior limbs with $\sim 5 \times 10^5$ C6 cells ($2.5 \times 10^6$ cells ml$^{-1}$), and then monitored daily for tumor growth. Mice were intra-tumorally (i.t.) injected with $100 \mu l$ ($10^{10}$ vp) Ad-BmK CT, Ad or A195 buffer every five days. Five mice model were used in each group. The volume of a tumor was measured three times every week by determining two perpendicular dimensions with calipers and tumor volume in cm$^3$ was estimated according to the following formula: $V = \pi \times a \times b^2/6$, where 'a' was the longer diameter and 'b' was the diameter at a right angle.

The handing of mice and experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences.

Experimental metastasis assays

$5 \times 10^5$ C6/RFP (red fluorescence protein) ($2.5 \times 10^6$ cells ml$^{-1}$) cells were implanted into the right anterior limbs of six-week-old female BALB/c allogeneic athymic nude mice. Mice were intratumorally (i.t.) injected with $100 \mu l$ ($10^{10}$ vp) Ad-BmK CT, Ad or A195 formulation buffer every five days. Five mice model were used in each group. Mice were killed and dissected at 50 days after tumor inoculation. The tumors and disordered lung tissues were sampled and fixed with 4 % paraformaldehyde in PBS. The C6/RFP cells of lung were imaged respectively. Paraffin sections and H&E staining were prepared following routine procedures to verify tumorigenesis pathologically.

Statistical analysis

All data was presented as the mean $\pm$ SD. The significance of the difference between groups was evaluated by one-way and two-way repeated measures ANOVA and multiple comparisons with SPSS 10.0 software. A value of $p < 0.05$ was considered statistically significant.

Results

Construction and purification of recombinant Ad-BmK CT

Restriction digestion of recombinant pAdEasy-BmK CT plasmid DNA with Pac I for 5 h at 37 °C yielded a large and small fragment of 23 and 4.5 kbp (Fig. 1a).
Fig. 3  Infection efficiency assay of the adenoviral vector to C6 cells. C6 cells were infected with recombinant Ad-BmK CT and control (Ad), the percentage of expressed GFP was analyzed via FCM. The percentage of infected cells increased rapidly between $5 \times 10^3$ and $5 \times 10^4$ vp cell$^{-1}$. a C6 cells were infected with Ad $5 \times 10^4$ vp cell$^{-1}$ for 48 h, the percentage of GFP positive cells reached the top. b Showed the same results post-injected with Ad-BmK CT. c showed the infection efficiency of Ad and Ad-BmK CT. Columns, mean the percentage of GFP positive cells; bars, SD. Date shown is representative of three independent experiments. ** the maximum percentage. d The maximum percentage of GFP positive cells were maintained morphologically by fluorescence microscopy. e Showed the level of GFP expression in C6 cells post-injected with Ad $2.5 \times 10^4$ vp cell$^{-1}$ for 148 h
To confirm the recombinant adenovirus, genomic DNA of the recombinant adenovirus pAdEasy-\textit{Bm}K CT was used as the template in PCR analysis. The group of comparison was pAdEasy-1 which did not contain any foreign gene. The products of PCR were indentified by 2 % agarose gel electrophoresis (Fig. 1b). The DNA sequence determination confirmed that pAdEasy-\textit{Bm}K was constructed correctly.

Adenovirus carrying \textit{Bm}K CT was produced in AD293 cells, and the progress of the transfection was monitored by fluorescence microscopy (Fig. 1c). The titer of Ad-\textit{Bm}K was approximately $2.5 \times 10^{12}$ vp ml$^{-1}$.

Detection of \textit{Bm}K CT in transfected AD293 cells or C6 cells

The \textit{Bm}K CT transgene expression in AD293 cells or C6 cells mediated by adenovirus transfection was determined by RT-PCR. A significant amount of \textit{Bm}K CT expression was found in Ad-\textit{Bm}K CT-tranfected AD293 cells (Fig. 2a). To determine the expression levels of \textit{Bm}K CT, the total RNA extracted from transfected C6 tumor cells at the indicated time ($12 \sim 72$ h) were subjected to RT-PCR. As shown in Fig. 2b, the amounts of \textit{Bm}K CT expression had always been stably maintained during these times.

Analysis of infection effect of adenoviral vector to C6 cells

Ad and Ad-\textit{Bm}K CT were used to test the ability of the adenoviral vector to infect C6 cells (Fig. 3a, b). The infection efficiency was determined by the percentage of GFP positive cells. The best infection effect was obvious in C6 cells at $5 \times 10^4$ vp cell$^{-1}$ for 48 h. The infection efficiency of Ad was $\sim 21\%$ at $5 \times 10^3$ vp cell$^{-1}$, $\sim 28\%$ at $10^4$ vp cell$^{-1}$, $\sim 72.5\%$ at $2.5 \times 10^4$ vp cell$^{-1}$, $\sim 91.5\%$ at $5 \times 10^4$ vp cell$^{-1}$; and that of Ad-\textit{Bm}K CT was $\sim 16\%$ at $5 \times 10^3$ vp cell$^{-1}$, $\sim 34\%$ at $1 \times 10^4$ vp cell$^{-1}$, $\sim 78.5\%$ at $2.5 \times 10^4$ vp cell$^{-1}$, $\sim 92.5\%$ at $5 \times 10^4$ vp cell$^{-1}$, respectively (Fig. 3c). The expression of GFP was observed by fluorescence microscopy after C6 cells were infected with Ad-\textit{Bm}K CT or Ad at $5 \times 10^4$ vp cell$^{-1}$ for 48 h (Fig. 3d). The C6 cells were infected with Ad at $2.5 \times 10^4$ vp cell$^{-1}$ for 148 h, briefly fixed with paraformaldehyde, stained with DAPI and then examined by fluorescence microscopy. As shown in Fig. 3e, more than 90 % of GFP expression was found in C6 cells infected with Ad at $2.5 \times 10^4$ vp cell$^{-1}$, but C6 cells were round and detached from the wall and no more than 10 % of C6 cells were alive after post-injection of Ad-\textit{Bm}K CT at $2.5 \times 10^4$ vp cell$^{-1}$ for 148 h (data not shown).

**Ad-\textit{Bm}K CT inhibits glioma growth in vitro**

MTT test was performed to investigate inhibition effect of recombinant adenovirus to glioma cells in vitro. In this assay, we assessed the potential therapeutic effects of recombinant Ad-\textit{Bm}K CT against rat glioma C6 cells. Firstly, the adenovirus is a good system to study \textit{Bm}K CT expression in glioma cells. On the other hand, adenovirus has inhibitory effects on glioma cell growth. The control of glioma growth was performed by \textit{Bm}K CT and adenovirus together. Figure 4 shows that Ad-\textit{Bm}K CT significantly inhibited the C6 cell growth in a time-dependent manner, compared to the Ad and control groups, indicating that transgene \textit{Bm}K CT expression suppressed C6 glioma cell proliferation in vitro. Based on these results, we postulate that adenoviruses may work synergistically with \textit{Bm}K CT in inhibiting glioma growth.

**Ad-\textit{Bm}K CT inhibits glioma growth in vivo**

To explore the anti-tumor effects of Ad-\textit{Bm}K CT in vivo, the glioma-bearing nude mice were intratumorally injected with $10^{10}$ vp Ad-\textit{Bm}K CT or Ad every five days, and the control was injected with A195 buffer of the same volume (100 µl). Tumor volume analysis suggested that compared with Ad and A195 buffer treatment groups, Ad-\textit{Bm}K CT significantly inhibited glioma growth at 24 days (Fig. 5a). As shown in Fig. 5b, after four treatments, tumor growth was significantly retarded in Ad-\textit{Bm}K CT treatment group, compared with those in the Ad and control groups. After the fourth treatment, one mouse died post-treated 24 days post-treatment in the Ad and control groups, respectively. Up to 40 days post-treatment, four mice
died in the control group, and two in Ad group. There were no deaths in Ad-\textit{BmK CT} group (Fig. 5c).

\textbf{Ad-\textit{BmK CT} inhibit metastasis of glioma in vivo}

All lungs from experimental BALB/c allogeneic athymic nude mice were dissected after 50 days of treatment (Fig. 6a). The tumor volumes in Ad-\textit{BmK CT} group were smaller than those in Ad treatment, which suggested that the metastasis inhibitory effects of Ad-\textit{BmK CT} in vivo were not due to Ad. The degree of lung metastasis between the Ad treatment groups and Ad-\textit{BmK CT} groups revealed striking differences. C6/RFP cells of lungs could be imaged by Xenogen IVIS Spectrum (Fig. 6b). Histological analysis confirmed that the lung samples from Ad treatment group exhibited typical morphologic characteristics of glioma (Fig. 6c).

\textbf{Discussion}

Adenovirus-based vectors can be used as delivery vectors for anti-glioma or apoptosis-inducing genes, such as HSV-thymidine kinase/ganciclovir gene (HSV-tk/GCV), \textit{p53} gene and inhibitor of growth family member 4 gene (ING4) (Perez-Cruet et al. 1994; Badie et al. 1995; Nashimoto et al. 2005; Fan et al. 2010). The efficacy of Adv-mediated HSV-tk/GCV gene therapy to treat brain tumors had been confirmed in a syngeneic glioma rat model (Zhao et al. 2007) and in a syngeneic BT4C rat malignant glioma model, which did not involve any significant host immune responses against the tumor (Tyynela et al. 2002). \textit{p53} protein is an apoptotic factor for glioma cells (Badie et al. 1995) and a study of Adv-mediated transduction of the inducing \textit{p53} gene showed that the gene could induce apoptosis in human malignant glioma cell (Kurozumi et al. 2004) and enhance the oncolytic effect on glioma (Mitlianga et al. 2006). ING4 could suppress the brain tumor growth and angiogenesis by associating with the p65 (RelA) subunit of NF-\textit{kB} (Garkavtsev et al. 2004). Further results showed hING4 mediated by adenovirus could significantly inhibit the growth of C6 cells by inducing apoptosis (Zhao et al. 2007).

As shown here, the intact expression of \textit{BmK CT} in glioma cells is crucial for the inhibition of glioma. Adenovirus is widely used in brain tumor gene therapy because it can produce persistent infections and the adenoviral vector-mediated gene transfer approach is the prospect of inducing tumoricidal activity (DeBin et al. 1993). In this study, a recombinant replication-defective adenovirus served as the vehicle to deliver
BmK CT gene to the C6 glioma cells. Since BmK CT can specifically bind and inhibit the enzymatic activity of MMP-2, the up-regulation of which is partially responsible for the elevated migration ability of glioma cells, we developed this delivery system that specifically targets BmK CT to C6 glioma cells. Our work provides a strong biotechnological implication that the recombinant adenovirus Ad-BmK CT may be a powerful treatment method for glioblastoma.

Acknowledgments This project is supported by grants from “National Natural Science Foundation of China (No. 31272100, 31071924)”, the “National High Technology Research and Development Program of China (863 Program, No. 2012AA020809)”, and “the Program for the Top Young Academic Leaders of Higher Learning Institutions of Shanxi”. We wish to thank Prof. Hong Tang (Institute of Biophysics, Chinese Academy of Sciences) for technical advice on the animal experiments. We will also like to thank Shuhua Zheng (University of Miami Miller School of Medicine) for his comments and grammar revision of the paper.

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