pEGFP-N1-mediated *BmK* CT expression suppresses the migration of glioma

Yuejun Fu · Yanmei Jiao · Na An · Aihua Liang

Received: 6 June 2012 / Accepted: 18 November 2012 / Published online: 15 December 2012
© Springer Science+Business Media Dordrecht 2012

Abstract Gliomas can diffuse into the normal brain and this invasion of glioma cells involves modification of receptor-mediated adhesive properties of tumor cells, degradation and remodeling of extracellular matrix by tumor-secreted metalloproteinase (MMPs) such as MMP-2, consequently creating an intercellular space for invasion of glioma cells. *BmK* CT, one of the key toxins in scorpion *Buthus martensii* Karsch venom, is a novel blocker of the chloride ion channel and MMP-2. In this report, a recombinant plasmid pEGFP-N1-*BmK* CT was constructed and characterized by in vitro studies. The results showed that pEGFP-N1 mediated *BmK* CT expression displayed a high activity in suppressing cell migration via MMP-2. The potential therapeutic effect of pEGFP-N1 mediated *BmK* CT against rat glioma C6 cells was assessed and its potential mechanism was elucidated. It represented an approach for developing a novel therapeutic agent—recombinant plasmid pEGFP-N1-*BmK* CT as an efficient and powerful adjuvant.

Keywords *BmK* CT · Glioma cells · Matrix metalloproteinase-2 · Migration

Introduction

Glioma is a highly invasive, rapidly spreading form of brain cancer that is resistant to surgical and medical treatment. Malignant gliomas are the main brain tumors notoriously resistant to currently available therapies, since they fail to undergo apoptosis upon anticancer treatment (Wang and Ji 2005). Traditional therapy of brain tumors includes surgery, radiation therapy, chemotherapy, and immunotherapy (Yin et al. 2007). Despite these treatments, the median survival time is less than 15 months (Demuth and Berens 2004). It's clear that some adjuvant treatment will lead to newer and more effective methods and strategies for brain tumor control in the future.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases involved in the degradation of the extracellular matrix (ECM). The expression level of MMPs correlates with the invasion of cancer cells because the degradation of the ECM is essential for tumor angiogenesis, invasion and metastasis (Gladson 1999; Shao et al. 2011; Li et al. 2011; Lee et al. 2011). Of note, MMP-2 is up-regulated in gliomas and related cancers, but its expression level is trivial in normal brain tissue (Deshane et al. 2003), which demonstrates that MMP-2 can be regarded as a potential key target for glioma suppression.

Chlorotoxin (Cltx), one of the key toxins in scorpion *Leiurus quinquestriatus* venom, has been shown to bind specifically to glioma cell surface as a specific chloride channel blocker (Shen et al. 2005;

Y. Fu (✉) · Y. Jiao · N. An · A. Liang
Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education,
Institute of Biotechnology, Shanxi University,
Taiyuan 030006, People's Republic of China
e-mail: yjfu@sxu.edu.cn

Yang et al. 2005; Burton et al. 2008). 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). A chlorotoxin-like peptide gene, *BmK* CT, was cloned and sequenced from the venom of *B. martensii* 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 channel and MMP-2 (Fu et al. 2007).

Compared with other vectors (such as viral or nanoparticle vehicles), many cationic lipids naturally occurring or synthesized have been used for gene transfer in the form of liposomes, which have the advantages of non-immunogenicity, simple production, plasmid protected against nuclease degradation, non-oncogenicity, etc. And cationic lipids as an effective alternative of viral vector, and cationic liposomes can be used for cell transfection in vivo and in vitro. Here, we developed a recombinant plasmid pEGFP-N1-*BmK* CT which was designed to bind and inhibit the activity of the MMP-2. The aim of this study was to assess the antitumor efficacy of *BmK* CT gene therapy and develop new therapeutic strategy for glioma cancer.

Materials and methods

Materials

Rat glioma C6 cells were kindly provided by Prof. Wang (Institute of Clinical Medicine, Renmin Hospital, Yungang Medical College, Shiyan, Hubei, China). Dulbecco's modified Eagle's medium (DMEM) was from GIBCO BRL (Life Technologies, Carlsbad, CA, USA). Calf serum was from Hangzhou Evergreen Corp. Lipofectamine 2000 was from Invitrogen (Life Technologies). All reagents used are of highest molecular grade.

Construction of expression plasmid (Fig. 1)

The sequence of *BmK* CT was cloned from pRSETc-*BmK* CT using forward primer (5'-AACTCGAGA TGTGCGGTCCGTGCTTC-3') and reverse primer (5'-CGCGGATCCGTAGTAGTAACGGTTGC-3').

To obtain the pEGFP-N1-*BmK* CT expression plasmid, the *BmK* CT sequence was cloned into the pEGFP-N1 plasmid with restriction enzymes *Xho* I and *Bam*H I.

Cell culture

Cell cultures were prepared and maintained according to standard cell culture procedures. The rat C6 glioma cells were cultured at 37 °C in a humidified atmosphere of 5 % CO₂ in DMEM supplemented with 10 % (v/v) heat-inactivated calf serum.

Transfection of recombinant plasmids

For transient transfection, cells were harvested by pancreatin digestion and seeded on microtiter plates. Transfection of plasmids (pEGFP-N1, pEGFP-N1-*BmK* CT) was performed with Lipofectamine 2000 according to manufacturer's instructions: for 96-well microtiter plates and 24-well microtiter plates, cells were incubated with 0.2 µg plasmid DNA and 0.5 µl Lipofectamine 2000 or 0.8 µg plasmid DNA and 2 µl Lipofectamine 2000, respectively. The pEGFP-N1 containing green fluorescent protein cDNA was used for the observation of transfection efficiency. After 24 h, the transfection efficiency was monitored with a FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Gelatin zymography assay

Gelatinolytic activity of MMP-2 and MMP-9 was analyzed by gelatin zymography assay. C6 cells were cultured in the 96-well microtiter plates and the cells transfected with recombinant plasmids were incubated for 24 h. Culture media were then changed to serum-free cell culture media. Serum-free media conditioned for 24 h were subjected to 10 % (v/v) SDS-PAGE containing 0.1 % gelatin. The gel was incubated twice (for 30 min at room temperature) in 2.5 % Triton X-100. The gel was then incubated in activation buffer (5 mM CaCl₂, 1 mM ZnCl₂, and 0.005 % Brij, 50 mM Tris/HCl, pH 8.0) at 37 °C overnight, stained with Coomassie Brilliant Blue R-250 and briefly destained in 10 % (v/v) acetic acid and 40 % (v/v) methanol. Gelatinolytic activity of MMPs was detected as transparent bands on the blue background.

Western blot analysis

An equal number of C6 glioma cells transfected with recombinant plasmids (pEGFP-N1, pEGFP-N1-*BmK* CT) were incubated for 24 h. The culture medium was removed. Then cells were washed gently three times with PBS and cultured for another 24 h with culture medium devoid of FBS. Protein was collected from the culture medium. The protein concentration was determined with BCA Protein Assay Kit (Beyotime, Haimen, Jiangsu, China), separated on a 10 % SDS-PAGE gel and then transferred to nitrocellulose membrane. The membrane was blocked and then probed with antibodies against MMP-2 (1:100) (Boster Biotech, Wuhan, China). Goat anti-rabbit IgG was used as the second antibody (1:1500). β -actin in cell lysis solution was probed as control.

In vitro wound healing assay

The effect of EGFP, EGFP-*BmK* CT on cell migration was examined using in vitro wound healing assay. C6 cells were cultured in the 24-well microtiter plates. After transfection with recombinant plasmids for 24 h, a ‘scratch’ was introduced by scraping the C6 cells monolayer with a P200 pipette tip and then washed gently three times with cell culture medium to remove cell debris. The cells were then cultured in serum-free culture medium. Percent migration rate (MR %) was calculated as: $MR \% = (1 - T/C) \times 100 \%$, where T and C represent, respectively, the scrape distance at the indicated treatment times and at the previous treatment time.

Fig. 1 Construction of expression plasmid pEGFP-N1-*BmK* CT. The physical and genetic maps of pEGFP-N1 are shown in this figure. The gene *BmK* CT was fused with EGFP via the *Xho* I/*Bam*HI linker

Results and discussion

Construction and transfection of expression plasmid pEGFP-N1-*BmK* CT

The over-expression of *BmK* CT in the cytoplasm is crucial for glioma inhibition in this report. In this study, pEGFP-N1 served as the vehicle to deliver an intact *BmK* CT gene and an imaging reporter gene to the C6 glioma cells. According to the construction of pEGFP-N1-*BmK* CT expression plasmid (Fig. 1), DNA sequence determination confirmed the correct fusion of linker and the target gene (*BmK* CT) in the vector pEGFP-N1. By counting green fluorescing cells with plasmid pEGFP-N1 transfection, the transfection efficiency of C6 cell line mediated with liposome was above 70 % at 24 h (Fig. 2). This high-efficiency transfection led to the high level of EGFP or EGFP-*BmK* CT needed to perform the next assays.

pEGFP-N1 mediated *BmK* CT inhibits the secretion level of pro-MMP-2

MMP-2 appeared to be a major matrix metallo-proteinase in gliomas and was absent from normal brain tissue. As shown in Fig. 3a, transparent bands of

pro-MMP-2 (~70 kDa), MMP-2 (~60 kDa), pro-MMP-9 (~90 kDa) and MMP-9 (~85 kDa) were detected in the gelatin zymography assay. The activity of pro-MMP-2 was suppressed in the EGFP-*BmK* CT treatment group compared to that in the EGFP and control groups, which demonstrated that, in the cytoplasm, EGFP-*BmK* CT could also suppress the secretion levels of MMP-2. At the same time, the activity of MMP-9 was suppressed appreciably in the EGFP-*BmK* CT treatment group since both MMP-2 and MMP-9 have a similar structure. Relative activity was calculated as T/C, where C and T represent, respectively, the densitometry values of pro-MMP-2 in the control group and pro-MMP-2,9 and MMP-2,9 in the treatment group (Fig. 3b). Western blot confirmed that the expression of MMP-2 was suppressed in the EGFP-*BmK* CT treatment group compared with that in the EGFP and control groups (Fig. 3c).

pEGFP-N1 mediated *BmK* CT expression suppresses the migration of glioma

Gelatinase MMP-2 is known to be related with tumor invasion and progression. So, MMP-2 is critical for C6 glioma cells migration and metastasis. As shown in Fig. 4b, the average migration rates of the pEGFP-N1-

Fig. 2 Photograph of the transfected cells sample obtained with a laser scanning confocal microscope, C6 cells were transfected with pEGFP-N1 and pEGFP-N1-*BmK* CT using Lipofectamine 2000™ reagent and cultured for 24 h in the absence of serum. All transfections were performed in 96 or 24 well plates. EGFP was excited at 488 nm with an argon laser and fluorescence was detected at 500–540 nm. Scale bar: 200 μm

Fig. 3 Effect of pEGFP-N1 mediated *BmK* CT expression on MMP-2, 9 activity in C6 glioma cells. **A** Gelatin zymographic analysis of the conditioned media collected from cells treated with EGFP and EGFP-*BmK* CT. Gelatinolytic activity of pro-MMP-9 (~90 kDa), pro-MMP-2 (~70 kDa), active-MMP-9 (~85 kDa), and active-MMP-2 (~60 kDa) was detected as four transparent bands on the blue background. The gel shown is

BmK CT transfection group were lower than of the control group and the pEGFP-N1 transfection group at 6, 12 and 24 h, respectively. Compared to these of EGFP treatment groups, migration rates of C6 glioma cells were suppressed by approx. 66, 76 and 23 % in the presence of EGFP-*BmK* CT at 6, 12 and 24 h, respectively. These results showed that pEGFP-N1 mediated *BmK* CT expression displayed a high activity in suppressing cell migration via MMP-2.

Glioma cancer is a problem of or a challenge to clinical treatment. More effective treatment strategies and methods are needed clinically. The combination of some routine treatment methods with new biological therapy may be promising. It has been documented that *BmK* CT can prevent the metastasis of glioma cells in vivo and ^{131}I -labeled or Cy5.5-conjugated *BmK* CT selectively targets glioma in situ (Fan et al. 2010). 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 tried to develop a

representative of three treatment groups. **B** Relative activity analysis of pro-MMP-9, pro-MMP-2, active-MMP-9 and active-MMP-2. Densitometry analysis of MMPs bands in gel was performed using Gene Tools 4.01.02 software (Synoptics Ltd) and relative activity was calculated using Sigma Plot 10.0 (Jandel Corp.) (Representative of three independent experiments.) **C** Western blot analysis of Pro-MMP-2 and MMP-2

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. In our previous study, site-directed mutagenesis of *BmK* CT, wound healing assay, gelatin zymography assay and computational simulation highlighted the importance of electrostatic contribution to *BmK* CT-MMP-2 catalytic domain complex and a model of *BmK* CT-MMP-2 catalytic domain complex was proposed (Fu et al. 2011). It was the first documentation of the structural mechanism of the inhibition of glioma cell migration by *BmK* CT. Subsequently, we developed a type of glioma-specific multifunctional fluorescent nanodiamonds (FND)—*BmK* CT which was designed to bind and inhibit the activity of the MMP-2 endopeptidase, and to induce endocytosis of the lipid rafts, subsequently limiting invasive cell activities (Fu et al. 2012).

Tumor-targeted delivery, biocompatibility and stability are technological challenges in the development of effective nanoparticle (NP)-based diagnostic and

Fig. 4 pEGFP-N1 mediated *BmK* CT expression inhibited C6 cell migration rate. **A** Migration of C6 cells was routinely monitored after confluent monolayer of C6 cells were gently scratched with a plastic pipette tip. Migration distance of C6 cells was detected at 6, 12, and 24 h. **B** Migration rate analysis showed that EGFP-*BmK* CT displayed a higher activity in inhibiting cell migration than EGFP and control did. (Representative of three independent experiments.) Error bars represent the standard deviation

therapeutic agents. Despite great progress in recent years, experimental animal studies may never accurately predict the outcome of human clinical trials. Several important questions still need to be answered with respect to the use of nanoparticle vehicles to treat gliomas, such as whether they are able to cross the blood brain barrier, their concentration in tumors, and their mechanism of action within the tumor. Also, the inorganic particles as well as carbon or silica NPs are non-biodegradable. So, these materials cannot be considered as a safe excipient for parenteral pharmaceutical formulations. Therefore, in this study, the potential therapeutic effect of pEGFP-N1 mediated *BmK* CT against rat glioma C6 cells was assessed in vitro and its potential mechanism was elucidated. The vectors and the gene transfecting methods are the main restrictions of tumor gene therapy. Clinically, liposome-mediated gene transfer is suitable. The findings presented in this work are essential for the further exploration of this gene therapy of glioma tumor.

Acknowledgments This project was 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.2012AA

020809)’, and ‘the Program for the Top Young Academic Leaders of Higher Learning Institutions of Shanxi’.

Conflict of interest The authors state that they have no conflict of interest.

References

- Burton JB, Johnson M, Sato M, Koh SB, Mulholland DJ, Stout D, Chatziioannou AF, Phelps ME, Wu H, Wu L (2008) Adenovirus-mediated gene expression imaging to directly detect sentinel lymph node metastasis of prostate cancer. *Nat Med* 14:882–888
- Demuth T, Berens ME (2004) Molecular mechanisms of glioma cell migration and invasion. *J Neurooncol* 70:217–228
- Deshane J, Garner CC, Sontheimer H (2003) Chlorotoxin inhibits glioma cell invasion via matrix metalloproteinase-2. *J Biol Chem* 278:4135–4144
- Fan S, Sun Z, Jiang D, Dai C, Ma Y, Zhao Z, Liu H, Wu Y, Cao Z, Li W (2010) *BmK* CT toxin inhibits glioma proliferation and tumor metastasis. *Cancer Lett* 291:158–166
- Fu YJ, Yin LT, Liang AH, Zhang CF, Wang W, Chai BF, Yang JY, Fan XJ (2007) Therapeutic potential of chlorotoxin-like neurotoxin from the Chinese scorpion for human gliomas. *Neurosci Lett* 412:62–67
- Fu YJ, An N, Chan KG, Wu YB, Zheng SH, Liang AH (2011) A model of *BmK* CT in inhibiting glioma cell migration via matrix metalloproteinase-2 from experimental and molecular dynamics simulation study. *Biotechnol Lett* 33:1309–1317

- Fu YJ, An N, Zheng SH, Liang AH, Li YQ (2012) BmK CT-conjugated fluorescence nanodiamond as potential glioma-targeted imaging and drug. *Diam Relat Mater* 21:73–76
- Gladson CL (1999) The extracellular matrix of gliomas: modulation of cell function. *J Neuropathol Exp Neurol* 58:1029–1040
- Lee SY, Park SY, Kim SH, Choi EC (2011) Expression of matrix metalloproteinases and their inhibition in squamous cell carcinoma of the tonsil and their clinical significance. *Clin Exp Otorhinolaryngol* 4:88–94
- Li LW, Chen P, Ling Y, Song XM, Lu ZJ, He QH, Li ZY, Lu N, Guo QL (2011) Inhibitory effects of GL-V9 on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-2/9. *Eur J Pharm Sci* 43:393–399
- Shao WL, Wang W, Xiong XG, Cao C, Yan TD, Chen GQ, Chen HZ, Yin WQ, Liu J, Gu YY, Mo MC, He JX (2011) Prognostic impact of MMP-2 and MMP-9 expression in pathologic stage IA non-small cell lung cancer. *J Surg Oncol* 104:841–846
- Shen S, Khazaeli MB, Gillespie GY, Alvarez VL (2005) Radiation dosimetry of I-chlorotoxin for targeted radiotherapy in glioma-bearing mice. *J Neurooncol* 71:113–119
- Wang WX, Ji YH (2005) Scorpion venom induces glioma cell apoptosis in vitro and inhibits glioma tumor growth in vivo. *J Neurooncol* 73:1–7
- Wu JJ, Li D, Lan ZD, Chi CW (2000) The gene cloning and sequencing of Bm-12, a Chlorotoxin-like peptide from the scorpion *Buthus martensii* Karsch. *Toxicon* 38:661–668
- Yang R, Peng F, Liu H, Cao ZJ, Li WX, Mao X, Jiang DH (2005) Functional analysis of a gene encoding a chlorotoxin-like peptide derived from scorpion toxin. *Chin J Biochem Mol Biol* 21:19–23
- Yin LT, Fu YJ, Xu QL, Yang J, Liu ZL, Liang AH, Fan XJ, Xu CG (2007) Potential biochemical therapy of glioma cancer. *Biochem Biophys Res Commun* 362:225–229
- Zeng XC, Li WX, Zhu SY, Peng F, Zhu ZH, Wu KL, Yang FH (2000) Cloning and characterization of a cDNA sequence encoding the precursor of a chlorotoxin-like peptide from the Chinese scorpion *Buthus martensii* Karsch. *Toxicon* 38:1009–1014