Combination of lithium chloride and pEGFP-N1-*BmK* CT effectively decreases proliferation and migration of C6 glioma cells

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Abstract Deleterious invasiveness of glioma cells into the normal brain tissue is endorsed by its inherent ability to regulate the receptor-mediated adhesive properties, extracellular matrix degradation and remodeling and elevated secretory ability of metalloproteinase (MMPs) such as MMP-2. By doing so, it will create an intercellular space for the invasion of glioma cells. Here, we reported that combination of gene therapy *Buthus martensii* Karsch (*BmK*) CT, a type of scorpion toxin peptide, with lithium chloride (LiCl), clinically used as mood stabilizer, could inhibit the migration and invasion of C6 glioma cells. The results showed that concomitant administration of LiCl and pEGFP-N1-*BmK* CT on glioma cells would hamper pro-MMP2 secretion and in the meantime, inhibited its proliferation in a synergistic manner. These results try to extrapolate the potential interplay

between the combined treatment of LiCl and *BmK* CT with signaling pathways β -catenin, MMP, GSK-3 in C6 glioma cells. This strategy can stand for a novel approach designated for the development of a new method for glioma therapy.

Keywords Glioma · *BmK* CT · Lithium chloride · Migration

Introduction

Glioma is the most common type of human brain tumors. Traditional therapy of brain tumors includes surgery, radiation therapy, chemotherapy, and immunotherapy (Yin et al. 2007). Despite these intense treatments, contemporary median survival time is still less than 15 months (Demuth and Berens 2004). It is clear that some adjuvant treatment will lead to newer and more effective methods and strategies for treating glioma.

BmK CT, isolated from the venom gland of *Buthus martensii* Karsch, is a 36 mer peptide cross-linked by four disulfide bridges. Its sequence identity is 68 % similar to CTX isolated from the scorpion *Leiurus quinquestriatus* (DeBin et al. 1993). *BmK* CT was characterized as a novel blocker of the chloride ion channel and matrix metalloproteinase-2 (MMP-2) of glioma cells (Fu et al. 2007). MMPs are a family of endopeptidases that have the capacity to degrade all components of the extracellular matrix. Tumor-

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secreted metalloproteinases (MMPs) such as MMP-2, can degrade and remodel of extracellular matrix and thus create an intercellular space for invasion of glioma cells. So MMP-2 activity is essential for the invasion of glioma cells (Deshane et al. 2003).

LiCl has been used clinically as therapeutic agents in treating bipolar disorder (Feinstein 1998; Kagaya et al. 2000) and was recently proven efficient in inhibiting glioma cell migration and invasion. Other reported work showed that a high concentration of LiCl (20 mM) was required to obtain significant inhibitory effects on the migration ability of glioma cell (Nowicki et al. 2008). But LiCl is often toxic at high concentrations, thus limiting its application as a therapeutic agent against gliomas (Markovic et al. 2005).

In our previous report, we confirmed that there were potential therapeutic benefits of the simultaneous administration of LiCl and *BmK* CT for the treatment of gliomas (Fu et al. 2012). In this study, the effect of LiCl and gene therapy pEGFP-N1-*BmK* CT on glioma cells was studied and we analyzed the mechanism that might explain the inhibitory effect of combination therapy on glioma migration.

Materials and methods

Materials

pEGFP-N1-*BmK* CT was constructed in our laboratory (Fu et al. 2013). Rat C6 glioma cells were kindly provided by Prof. Wang (Institute of Clinical Medicine, Renmin Hospital, Yungang Medical College, Shiyang, Hubei, China). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco BRL. Calf serum was from Hangzhou Evergreen Corp. (Hangzhou, China). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). Gelatin and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rabbit-anti β -catenin antibody was purchased from Wuhan Boster Biological Technology Corp (Wuhan, China). Rabbit-anti-rat β -actin was purchased from Sangon Biotech Corp (Shanghai, China). All reagents used were of highest molecular grade.

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

C6 glioma cells were cultured in the 6-, 24-, and 96-well microtiter plates for Western blot assay, colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay, gelatin zymography assay and in vitro wound healing assay, respectively. For transient transfection, cells were harvested by pancreatic digestion and seeded on microtiter plates. Transfection of plasmids (pEGFP-N1, pEGFP-N1-*BmK* CT) was performed with Lipofectamine 2000 according to the manufacturer's instructions: for 6-well microtiter plates, cells were incubated overnight with 4.0 μ g of plasmid DNA and 10 μ l of Lipofectamine 2000 (Invitrogen) in Opti-MEM. Similarly, for 96-well microtiter plates and 24-well microtiter plates, cells were incubated with 0.2 μ g of plasmid DNA and 0.5 μ l Lipofectamine 2000 and 0.8 μ g of plasmid DNA and 2 μ l of Lipofectamine 2000, respectively.

MTT assay

The effect of LiCl on cell growth was examined by MTT assay. This assay is based on the cellular conversion of a tetrazolium salt into a formazan product that was easily detected using 96-well plate reader. Therefore, the MTT assay is a simple and sensitive method to study cellular survival and cellular growth, cytotoxicity, cell attachment and apoptosis. Equivalent number of C6 glioma cells (10^4 cells well⁻¹) was added to a 96-well microtiter plate, followed by adding the medium to give the final volume of 100 μ l. Then, the untreated cells served as control, or cells were transfected with pEGFP-N1, pEGFP-N1-*BmK* CT and treated with LiCl for 24 h, respectively. Then tetrazolium salt was added to each well and the cells were further incubated for 4 h, the media were discarded and DMSO (150 μ l) was added to each well. The absorbance at 490 nm of each well was recorded using a microplate reader (Bio-Rad 550, Hercules, CA, USA). Inhibition rate (IR) was calculated according to the following equation: $IR (\%) = (C - T)/C \times 100 \%$, where T and C represented the mean optical density of the treated sample and

the control, respectively. Experiments were performed three times and the average values $\pm$ SD were recorded.

Gelatin zymography assay

Gelatinolytic activity of MMP-2 was analyzed by gelatin zymography assay. C6 cells transfected with recombinant plasmids (pEGFP-N1, pEGFP-N1-*BmK* CT) were incubated for 24 h and then treated with LiCl for 24 h. Culture media were then replaced by serum-free cell culture media. Serum-free media conditioned for 24 h were subjected to 10 % (v/v) SDS-PAGE (containing 0.1 % gelatin) electrophoresis analysis. 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 CaCl_2 , 1 mM ZnCl_2 , 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.

Cell harvesting and protein fractionation

Preparation of total and nuclear protein was conducted as previously described. Briefly, the transfectants harboring pEGFP-N1, pEGFP-N1-*BmK* CT were treated with LiCl for 24 h to prepare whole-cell lysates by centrifugation and lysed in 100 μ l of RIPA buffer (Sangon Biotech, Shanghai, China). For nuclear protein extraction, cells were washed with PBS, resuspended in 350 μ l of buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 1.5 mM Phenylmethanesulfonyl fluoride (Sangon Biotechn Corp, Shanghai, China), and incubated on ice for 15 min. Then NP-40 was added to a final concentration of 1 % (v/v) and vortexed for 1 min, followed by centrifugation for 10 min at 4 °C at $1,600\times g$. The supernatant of the cytosolic fraction was transferred to a new tube. The nuclear pellet was washed with 400 μ l of buffer A, pelleted for 10 min at 4 °C at $1,600\times g$ and then solubilized by addition of one pellet volume of NE buffer (20 mM Tris, pH 8.0, 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 25 % glycerol, and 1.5 mM Phenylmethanesulfonyl fluoride (Sangon Biotechn Corp, Shanghai, China). After that, one-fourth pellet volume of NaCl (5 M) and one pellet volume of NE buffer were added and then vortexed.

Western blot analysis

The protein concentration was determined by BCA assay and the samples were analyzed by electrophoresis using SDS-PAGE gel (10 % w/v). Western blot analysis was conducted by blotting onto the nitrocellulose membrane (Millipore, Billerica, MA, USA). Blots were blocked, incubated with the primary antibodies (anti- β -catenin (Wuhan Boster Biological Technology, China), 1:100 or anti- β -actin (Sangon Biotech, China), 1:500), and washed three times with PBS buffer containing 0.04 % (v/v) Tween-20. Blots were incubated with secondary antibody goat anti-rabbit IgG-HRP (Boster Biological Technology, Wuhan, China) (diluted at 1:2,000) for 1 h, washed with PBS buffer containing 0.04 % (v/v) Tween-20 three times and then developed using ECL kit (Amersham, Pittsburgh, PA, USA).

In vitro wound healing assay

The effect of LiCl on C6 glioma cell migration was examined using in vitro wound healing assay. After transfection with recombinant plasmids for 24 h and treated with LiCl for 24 h, a ‘scratch’ was introduced by scraping the C6 glioma cells monolayer with a sterile 200 μ l pipette tip and then washed gently twice with cell culture medium to remove cell debris. The cells were then cultured by serum-free culture medium. Percent migration rate (MR %) was calculated as: $\text{MR \%} = (1 - T/C) \times 100 \%$, where T and C represent the scrape distance at the indicated treatment times and at the previous treatment time, respectively.

Results

Lithium chloride improved the inhibition of proliferation of pEGFP-N1-*BmK* CT-transfected glioma cells

MTT assay was performed to determine the effect of LiCl on tumor growth. In general, LiCl, an inhibitor of GSK-3 β , could inhibit the cell proliferative activity. As shown in Fig. 1, after transfection with pEGFP-N1-*BmK* CT for 24 h and treatment with LiCl for 24 h, the growth inhibitory effects of pEGFP-N1-*BmK* CT was enhanced by LiCl treatment. In both assays, the inhibition rate of the combination treatment of

Fig. 1 Effect of lithium chloride and pEGFP-N1-*BmK* CT on C6 glioma cells proliferation. C6 glioma cells were transfected with pEGFP-N1, pEGFP-N1-*BmK* CT and then treated with 0–50 mM LiCl. Proliferation assay was performed by MTT test as described in the text. The inhibition rates of each group were calculated by recording the absorbance at 490 nm. This figure shows that LiCl inhibited the survival of C6 glioma cells in a dose-dependent manner in both groups. For each experiment, at least three independent transfection experiments were performed. Data are expressed as mean $\pm$ SD ($n = 3$). P values less than 0.05 were considered statistically significant. * $P < 0.05$ for comparisons to pEGFP-N1 transfection groups, respectively

pEGFP-N1-*BmK* CT and LiCl were elevated compared with that of pEGFP-N1 and LiCl treatment. We proposed that *BmK* CT could, to some degree, diminish the side effects of LiCl in curbing the metastatic spread of glioma cells.

Effect of combination treatment on pro-MMP-2 activity in C6 glioma cells

As shown in Fig. 2, pro-MMP-2 was detected in the gelatin zymography assay. In the EGFP transfection group, the secretion level of pro-MMP-2 was gradually elevated by the treatment with LiCl, and this elevation was depended on the LiCl concentration. The activity of pro-MMP-2 was suppressed in the EGFP-*BmK* CT and LiCl treatment group. According to our previous work, we presumed that LiCl induced

inactivation of GSK-3 β could activate the Wnt/ β -catenin pathway, which would induce the expression of MMP-2 (Fu et al. 2014).

Combination treatment inhibited C6 glioma cells migration

MMP-2 and -9 have been hypothesized to be key enzymes responsible for the degradation of type IV collagen, the main component of extracellular matrix (ECM). These two enzymes may be critical for C6 glioma cells migration or metastasis. As shown in Fig. 3, migration rates of C6 glioma cells were suppressed in the EGFP and EGFP-*BmK* CT transfection groups by treatment with LiCl and the inhibition rate in the EGFP-*BmK* CT and LiCl treatment group was higher than the other treatment group.

Lithium chloride improved the accumulation of β -catenin in C6 glioma cell nucleus

As shown in Fig. 4, after transfection with recombinant plasmids for 24 h and treatment with LiCl for 24 h, the accumulation of β -catenin in the cell nucleus was elevated, and the accumulation of β -catenin was higher in the LiCl-treated C6 cells harboring EGFP-*BmK* CT than that in the C6 cells harboring EGFP.

Discussion

Glioma cancer remains a perplexing challenge for efficient treatment. More effective therapeutic strategies and methods are needed clinically. *BmK* CT, one of the key toxins in the scorpion *Buthus martensii* Karsch venom, is a novel blocker of the chloride ion channel and MMP-2 of glioma cells. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteases involved in the degradation of the extracellular matrix (ECM). LiCl has been used

Fig. 2 Effect of lithium chloride and pEGFP-N1-*BmK* CT on pro-MMP-2 activities in C6 glioma cells. Gelatin zymographic analysis of the conditioned media collected from treated cells.

Gelatinolytic activity of pro-MMP-2 was detected as one transparent band on the blue background. The gel shown is a representative result of two treatment groups

Fig. 3 Lithium chloride and pEGFP-N1-*BmK* CT inhibited C6 glioma cells migration. Migration distance of two transfection groups was detected at 6, 12 and 24 h after treating with 0–50 mM LiCl, respectively. Migration rate analysis showed that LiCl inhibited C6 glioma cells migration over 24 h. For each experiment, at least three independent transfection experiments were performed. Data are expressed as mean $\pm$ SD ($n = 3$). Statistics were performed between the pEGFP-N1 and pEGFP-N1 + LiCl Treatment, pEGFP-N1-*BmK* CT and pEGFP-N1-*BmK* CT + LiCl Treatment at 6, 12 and 24 h, respectively. P values less than 0.05 were considered statistically significant

Fig. 4 Effect of lithium chloride and pEGFP-N1-*BmK* CT on the accumulation of β -catenin in the cell nucleus. C6 glioma cells were transfected with pEGFP-N1, pEGFP-N1-*BmK* CT and treated with 0–50 mM LiCl, respectively. Western blot analysis showed that LiCl improved the accumulation of β -catenin in cell nucleus

clinically for 50 years in the treatment of bipolar disorder. Inhibition of GSK-3 β has recently been proven to be efficient in inhibiting glioma cell. In this study, we found that pEGFP-N1-*BmK* CT and LiCl

had complementary effects towards the inhibition of the secretion level of pro-MMP-2 and proliferation of C6 glioma cells. When C6 cells were treated by LiCl, the accumulation of β -catenin in the cell nucleus was increased in pEGFP-N1 group and pEGFP-N1-*BmK* CT group.

Transfection of pEGFP-N1-*BmK* CT into glioma cells will supposedly interact and inhibit the intracellular MMP2. So far, anti-tumor efficiency of all CTX-like small peptides was evaluated in extracellular models, either by adding these peptides to culture medium or injecting into the circulation system of animal models. For the first time, the potential effect of intracellular expression of CTX-like peptide was evaluated and low side effects combination strategy was proposed in this paper. Lithium chloride is the therapeutic agent that has been safely used in clinics for more than 50 years, indicating it is well tolerated by patients. Targeted gene therapy is a future strategy for cancer treatment and promising clinical trials are ongoing using different vectors and genes they are carrying. Based on the results in this paper, we will further screen the best vector for the future in vivo delivery of the gene encoding *BmK* CT. By doing so, the clinical relevance of this project will be further highlighted. Also, our results implicate the impact of LiCl, *BmK* CT concomitant treatment on β -catenin, MMP, GSK-3 signaling pathways in glioma cells. The results are consistent with our previous report regarding how addition of *BmK* CT protein will have synergistic cytotoxic effects towards C6 glioma cells (Fu et al. 2012). To sum up, these results indicated the antitumor efficacy of LiCl and *BmK* CT gene therapy and might provide a new therapeutic strategy for glioma cancer.

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