

Quantitative analysis of U251MG human glioma cells invasion in organotypic brain slice co-cultures

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Abstract. – OBJECTIVE: To develop an *in vitro* model under conditions that highly resemble the *in vivo* situation for searching new therapeutics targeting invasive glioma cells.

MATERIALS AND METHODS: We generated organotypic brain slice “co-cultures” (OBSC) from mice and cultured the models on Millipore membrane inserts. U251MG glioma cells expressing enhanced green fluorescent protein (EGFP) were established. After cultured the glioma cells to form spheroids, we implanted the spheroids onto brain slice surface. Then we evaluated the invasion area and cell density after U251MG cells were treated with the Na⁺-K⁺-2Cl⁻ cotransporter 1 (NKCC1) inhibitor bumetanide by confocal laser microscopy.

RESULTS: In the models, the organotypic morphology and neuronal viability were well preserved. The confocal results showed that the cell spheroid area and density of U251MG cells in bumetanide group were decreased compared to the control group in brain slices. Meanwhile, the phospho-NKCC1(p-NKCC1) protein level of U251MG cells in bumetanide-treated group was also lower than the control group.

CONCLUSIONS: The OBSC model is a reliable and easy-to-perform *in vitro* method to quantify the glioma invasion ability.

Key Words:

Glioma, Invasion, Organotypic brain slice “co-culture”, Na⁺-K⁺-2Cl⁻ cotransporter1.

Introduction

Among central nervous system (CNS), glioma is one of the most common primary tumors. Current therapy strategy includes surgery, radiation and chemotherapy. Glioblastoma is the most malignant type of glioma with the median survival is only 14.6 months¹. People who suffer from it exhibit poor prognosis and relatively short median survival time, which are mainly due to its abil-

ity to invade the surrounding normal brain tissue regarded as one of its most lethal properties. To control such a lethal growth of glioma, better understanding the molecular and cellular mechanisms underlying the tumor invasiveness seems to be extremely essential. Thus, most common treatment strategies of glioma focus on intervening the invasion of tumor cells²⁻⁴.

Up to now, aiming to evaluate the intensity of glioma invasion, many experimental approaches have been employed, such as the Matrigel, wound healing assay, and tumor spheroid “co-culture” model⁵. However, these means are not appropriate enough for a study of invasion, for basement membranes do not actually constitute a barrier structure for the invading glioma cells in brains *in situ*. Moreover, the *ex vivo* assays are lack of cellular complexity and organotypic microenvironment^{5,6}. It is necessary to develop “co-culture” models to study the motility of glioma cells, and to monitor the cellular morphology, movement direction, migration area and invasion rate. Here, we describe the establishment of the organotypic brain slice “co-culture” (OBSC) presenting a robust and reliable tool to monitor glioma cell invasion. According to some literatures, slice cultures can be obtained from almost any portion of the brain⁷ or even from peripheral organs like kidneys, liver and others⁸. With this model which highly simulates the *in vivo* situation, we can study the invasion pathways of glioma cells. Since U251MG cell line is one of the most well characterized cell lines with a wide range of biological information available⁹, we labeled U251MG cell line with green fluorescent protein (GFP) to enhance the visualization of glioma cell invasion in the OBSC model.

With this model, we can demonstrate the invasion ability of glioma cells and studied the anti-invasion of glioma cells. Na⁺-K⁺-2Cl⁻ co-transporter 1(NKCC1), an important member of ion transporting systems in neuronal cells, was defined for its role in the

regulation of inward Na^+ - K^+ - 2Cl^- cotransport¹⁰. The role of NKCC1 had been extensively investigated in cancers and neurological diseases. NKCC1 high expression predicted a bad clinical outcome for lung adenocarcinoma patients¹¹. Otherwise, NKCC1 can modulate glioma cell migration and invasion, and the protein expression levels were significantly higher in glioma tissues than the normal brain¹²⁻¹⁴. Meanwhile, pharmacologic inhibition using the NKCC1 inhibitor bumetanide inhibits *in vitro* glioma cell migration and invasion¹².

In the present study, we implanted U251MG glioma cell spheroids on the surface of brain slices after treated by the NKCC1 inhibitor. We modified the invasion model by introducing confocal laser scanning microscopy (CLSM) so that three-dimensional evaluation and quantitative assessment of the process of glioma cell invasion in alive situations is available.

Compared with the control group, the inhibition of NKCC1 activity could impede cell invasion. This “co-culture” system enables us to choose a robust tool for experimental drug investigation of anti-glioma cell invasion which mimics *in vivo*.

Materials and Methods

Glioma cell culture

Human glioma cell line, U251MG, was purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, the Chinese Academy of Science, and the cells were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM, high-glucose) supplemented with 10% fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA) at 37°C in an atmosphere of 5% CO_2 and 95% room air. The medium was changed every other day, and the cells were treated with routine enzymatic digestion and passage.

U251MG Cell Line Transfection and Production of Glioma Spheroids

U251MG cells were transfected with the pEG-FP-N3 plasmid (Clontech, Mountain View, CA, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). This procedure was according to the manufacturer's instructions. Using cloning cylinder method to sort the stably transfected cells and G418 (Keygen Biotech, Nanjing, China) to enrich. The transfected cell clones were measured by 488 nm excitation by fluorescence microscopy (IX71 Olympus, Tokyo, Japan). And EGFP-expressing cells (5×10^5) were seeded into a

1.25% agar-coated flask (25 cm^2 , Costa, Corning, NY, USA) and left to incubate at 37°C under the standard conditions of 5% CO_2 and 95% air for three days. The U251MG glioma cell spheroids obtained with this method reached a diameter ranging from 100-300 μm . Cell spheroids with a diameter of around 150 μm were selected for the following experiments.

Preparation of Organotypic Slice Cultures

Brain slices were prepared according to the methods described previously^{15,16}. In this model, brain slices are maintained in culture at the interface between air and culture medium^{17,18}. In brief, C57BL/6 which was provided by the Laboratory Animal Center of China Medicine University (SPF grade) at postnatal days 3-4. All animal experiments were in accordance with the guidelines of the China Medical University Committee for Ethics of Animal Experimentation. The mice were decapitated with large scissors; then, the brain was quickly dissected out of the skull. After removal of the frontal and occipital poles including the cerebellum, the specimens were placed in Hank's balanced salt solution (HBSS) (Hyclone, Logan, UT, USA) and kept at 4°C with pH 7.35. Using Leica vibrating microtome (VT 1000S Leica, Nussloch, Germany), the specimen was cut vertically to the base in 400 μm thickness. Setting the frequency of 10 (100HZ) and speed of 0.2 (0.01 mm/s), the first several slices were abandoned until the basal ganglia displayed its typical tissue architecture. Four to six slices with intact basal ganglia cytoarchitecture were collected, then transferred to cell culture inserts (pore size 0.4 μm , Millicell-CM, Millipore, MA, USA) that was placed in six-well culture dishes (Corning, NY, USA). After the transfer, feeding them with 1ml culture medium consisting of 50% minimum essential medium (MEM), 24% horse serum, 25% HBSS, 1% penicillin-streptomycin (all from Hyclone, Logan, UT, USA) and supplemented with 36 mM glucose and 25 mM Hepes (Sigma, St. Louis, MO, USA). In order to acquire a relatively long-term survival of the neuronal cells, keeping the surface of the brain slice exposed to air was important. Slice cultures were incubated at 37°C under conditions of 5% CO_2 and 95% room air. Next day, the culture medium was replaced with fresh medium without antibiotics.

Assessment of Neuronal Viability of Brain Slices

To make sure the brain slice “co-cultures” were available to experiment, morphological observation

of “co-cultures” was performed every day using phase contrast microscope (DMI 3000B Leica, Wetzlar, Germany). For neuronal viability assessment, according to cellular absorption of propidine iodide (PI) before and after treatment with N-methyl-D-aspartate (NMDA) (Sigma, St. Louis, MO, USA). PI was dissolved in solution without serum, containing 75% MEM, 25% HBSS, 2 mM L-glutamine and 6.5 mg/ml glucose reach a final concentration of 4.6 $\mu\text{g}/\text{ml}$. As for the NMDA insult, after treated by 100 μM NMDA for 15 minutes, “co-cultures” were incubated with PI for 24 hours to assess neuronal inactivation. Finally, PI signals were viewed under an invert fluorescence microscope (IX71 Olympus, Tokyo, Japan) with a TRITC filter.

Invasion Assay in Brain Slice Model

Transferring spheroids with a diameter of approximately 150 μm into 3 days old brain slice cultures between the cortex and striatum into the corpus callosum by a microsyringe. The brain slice must not be damaged during the spheroids implantation. For quantitative analysis the biochemical reaction in the process of glioma invasion, we used confocal laser scanning microscope (FV1000 Olympus, Tokyo, Japan). To inhibit NKCC1, bumetanide was made to a concentration of 50 $\mu\text{mol}/\text{L}$ in PBS (Hyclone, Logan, UT, USA). Then 500 μL bumetanide solution was applied to the glioma spheroids on slice “co-cultures” every day. For control, PBS only was applied to glioma spheroids on other “co-cultures”. The brain slices, each containing one spheroid per slice culture, were incubated under standard conditions of 5% CO_2 and 95% room air at 37°C. The medium was changed every other day.

Assessment of Tumor Cell Invasion Using Confocal Microscopy

The tumor cell invasion was followed using a confocal laser scanning microscope with perfect focus system 6 hours after implantation as well as 3 and 6 days after implantation. The software FV1000 Viewer (Ver. 3.0 Olympus, Tokyo, Japan) was used for obtaining confocal Z-stacks. We placed the level of the basal plane (0 μm) on the surface of the brain slice and obtained pictures every 20 μm down through the “co-culture”. The images in the Z-stack were superimposed into one image and the total tumor cell invasion was assessed by FV1000 Viewer. Invasive glioma cells were counted in a zone of 300 μm from the center of spheroids and area of the spheroids were estimated.

Western Blot Analysis

Cell lysates were prepared by extracting proteins with RIPA buffer supplemented with phosphatase and protease inhibitors (both from Keygen Biotech, JiangSu, China). Protein concentrations were determined with a BCA protein assay kit (Keygen Biotech, JiangSu, China). Equal amounts of protein were separated by 8% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Indianapolis, IN, USA). After blocked with BSA, the PVDF membranes were rinsed briefly in TBST and incubated with primary antibodies at 4°C overnight. The blots were probed with total NKCC1 monoclonal antibody (1:2000, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and phospho-NKCC1 (p-NKCC1) polyclonal antibody (1:1000, Millipore, Billerica, MA, USA). Specific protein expression was then detected by incubating the washed membranes with HRP conjugated secondary antibodies. Protein bands were visualized by using the ECL Western blotting chemiluminescent detection reagents (Apexbio, Houston, TX, USA) and densitometrically quantitated according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA)

Statistical Analysis

Data are analyzed by analysis of variance (ANOVA) using SPSS 20.0 statistical software (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at the level of $p < 0.05$. Results are expressed as mean \pm standard deviation.

Results

The Brain Slice “co-culture” Procedure and Neuronal Viability

Here, we analyzed the OBSC to study glioma invasion, the procedure of brain slices section was shown in Figure 1. The tissue for this assay was derived from 3 days old C57BL/6 mice. Brains were cut into 400 μm thick organotypic slices and transferred onto transmembrane inserts for morphology and viability assessment. The cytoarchitecture containing the laminar structure of cortex (Figure 2A) and corpus callosum (Figure 2B) in the brain slices remained well preserved for over 2 weeks. The brain slices on insert membranes were thinning during one week of static culture, with the thickness reduced from 400

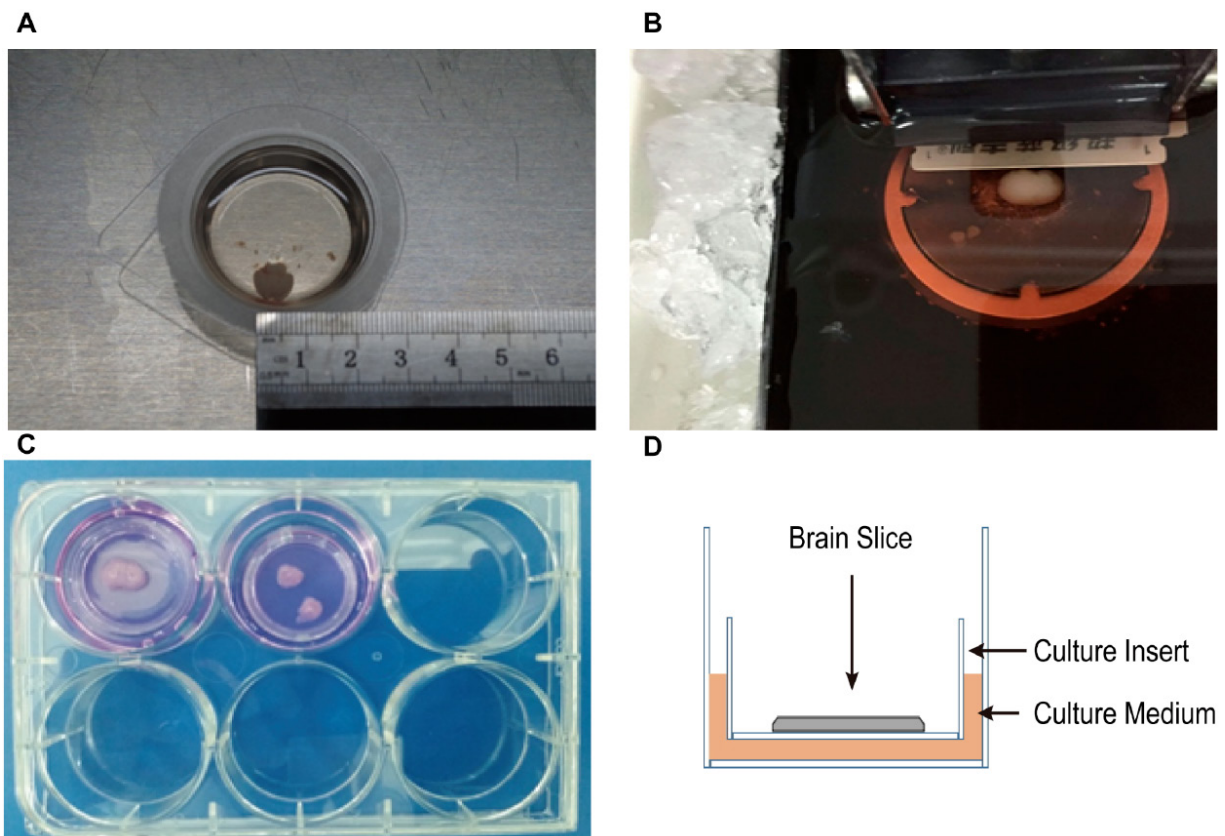


Figure 1. Procedure of brain slices section. **A**, The brain was quickly dissected out of the skull. **B**, Sectioning horizontal brain slices in 400 μm thickness by vibrating microtome. **C**, The brain slices were transferred onto the porous membrane of culture inserts place in a 6-well plate. Culture medium was added to the bottom of the culture well. **D**, The cross section of the “co-culture” system.

μm to 200 μm . We use PI staining to evaluate the neuronal viability of brain models before and after NMDA treatment (Figure 2C and 2D). Exposure to 100 μM NMDA for 15 minutes induced intense PI fluorescence in the brain slices (Figure 2D), which indicated the neuronal viability remained well.

Bumetanide Inhibits the U251MG Cell Invasion

After obtained U251MG spheroids with a diameter ranging 100-300 μm , we selected spheroids with a diameter of 150 μm and successfully implanted them into corticostriatal brain slice cultures. Serial sections were obtained every 5 μm downward from the brain slice surface to the bottom. At day 5 after implantation, we found the spheroids moved from the surface plane to deeper part, five representative sections are shown every 20 μm (Figure 3A-E), and the glioma cells investigated on the top (Figure 3A) and the bottom (Figure 3E) were fewer than other sections (Figure 3C and 3D). To represent one

spheroid, the individual images in the Z-stack were superimposed into one image, and the total tumor cell invasion was estimated in a zone of 300 μm around the center of the spheroid (Figure 3F).

To assess the glioma cells' invasion, we examined the distribution area and density of the EGFP labeled glioma cells using confocal laser microscopy. Confocal Z-stacks were obtained at 6 hours, day 3 and day 6 after implantation (Figure 4A-F). The area of the U251MG spheroids ($n=3$) increased rapidly over time (Figure 4D-F). In the “co-culture” system treated with bumetanide, the spheroid area and density increased slightly (Figure 4A-C). In the control group treated with PBS, the spheroid area and density increased apparently over time (Figure 4D-F). When U251MG spheroids treated with bumetanide, the micrographs of day 3 and day 6 after spheroids implantation show the invasion area and also the density of invasive cells reduced, compared to the control group (Figure 5).

Bumetanide Inhibits the Expression of p-NKCC1

NKCC1 phosphorylation stimulates NKCC1 activity to regulate the glioma cells invasion¹⁹. In our study, phospho-NKCC1 (p-NKCC1) protein levels were clearly decreased by bumetanide treatment (Figure 6A). As shown in Figure 6, in bumetanide group the relative p-NKCC1 protein level was $22.48 \pm 2.3\%$, which was significantly lower than $74.65 \pm 3.1\%$ in the control group (Figure 6B). These data indicated that the p-NKCC1 expression level in U251MG cells was clearly inhibited by treatment with bumetanide.

Discussion

In this study, we describe the establishment and evaluation of an *in vivo*-like invasion model, where human glioma-derived cell line was implanted into corticostriatal mice brain slice

“co-cultures”. It is based on the transwell interface method^{16,20,21} and has been previously tested in proof-of-principle assays, showing its suitability for remaining an organotypic environment with preserved extracellular complexity and neurovascular units^{22,23}. Also, the “co-culture” system can be acquired almost from any part of the brain⁷ or even from peripheral organs like kidneys, liver and others⁸. Tumor growth, optical and biochemical assessment of the microenvironment can be conducted by labeling with the fluorescent dye or implanting reporter gene expressing glioma cells (i.e. Fluorescent protein expression such as GFP, RFP and others), which is direct and efficient. Another advantage of this *ex vivo* model is that it enables pharmacological testing of virtually any drug compound in real time mode. These drugs could target at the tumor invasion, migration, angiogenesis²⁴ and tumor microenvironment^{25,26}.

Tumor invasion is a complex process in which tumor cells initiate migration from the primary site of

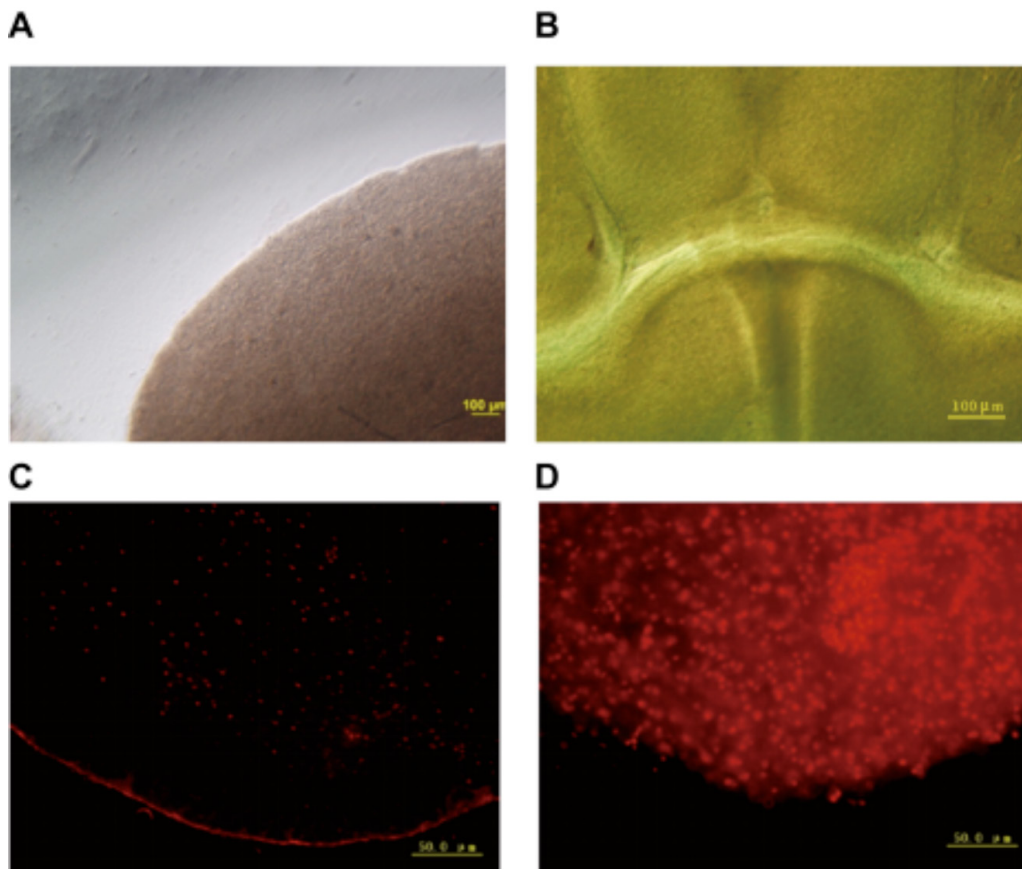


Figure 2. Morphology and viability of brain slices. **A**, Morphology and cytoarchitecture of the cortex on day 6. **B**, Morphology and cytoarchitecture of the corpus callosum on day 6. **C-D**, PI staining and intensity measurement in the brain slices before (C) and after (D) treatment with NMDA.

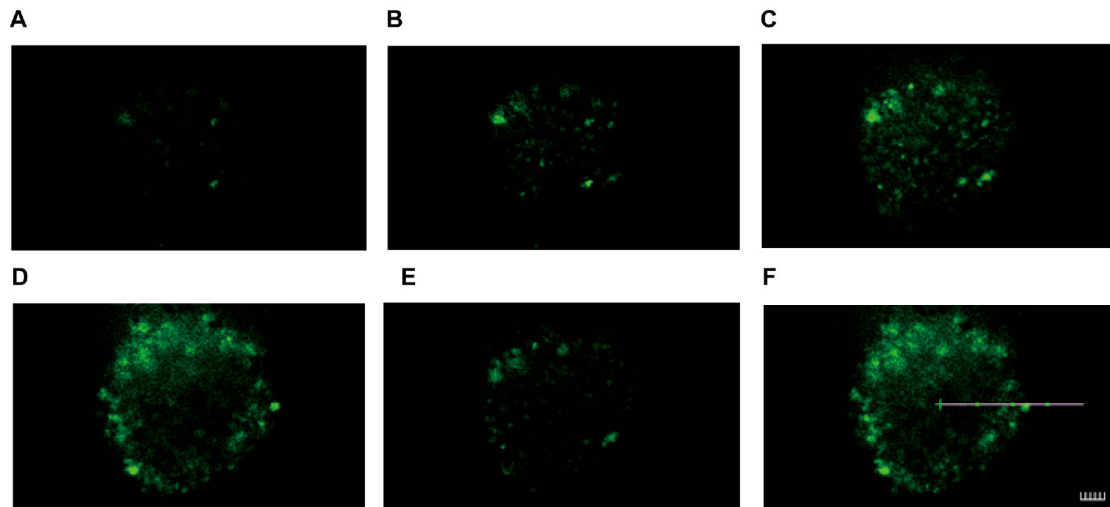


Figure 3. Confocal fluorescence micrographs show invasion of U251MG cells on day 6 after glioma spheroids implantation. **A-E**, Sections were obtained every 20 μm downward from the brain slice surface (0 μm) to a deeper section (100 μm). **F**, The images in the Z-stack were superimposed into one image. Invasive tumor cells were counted from the center of spheroids in a 300 μm zone. The spheroids area and tumor cell invasion were estimated. Scalebar 100 μm .

the tumor, adhere to the extracellular matrix (ECM). Therefore, in situ studies of glioma invasion require special conditions analogous to those of normal brain. We have established a novel brain slice model of glioma

tumor cell invasion by modifying an organotypic culture of brain tissues^{27,28}. Here, we were able to follow and quantify the spheroid area and tumor cell invasion into living brain slice “co-cultures” over time by

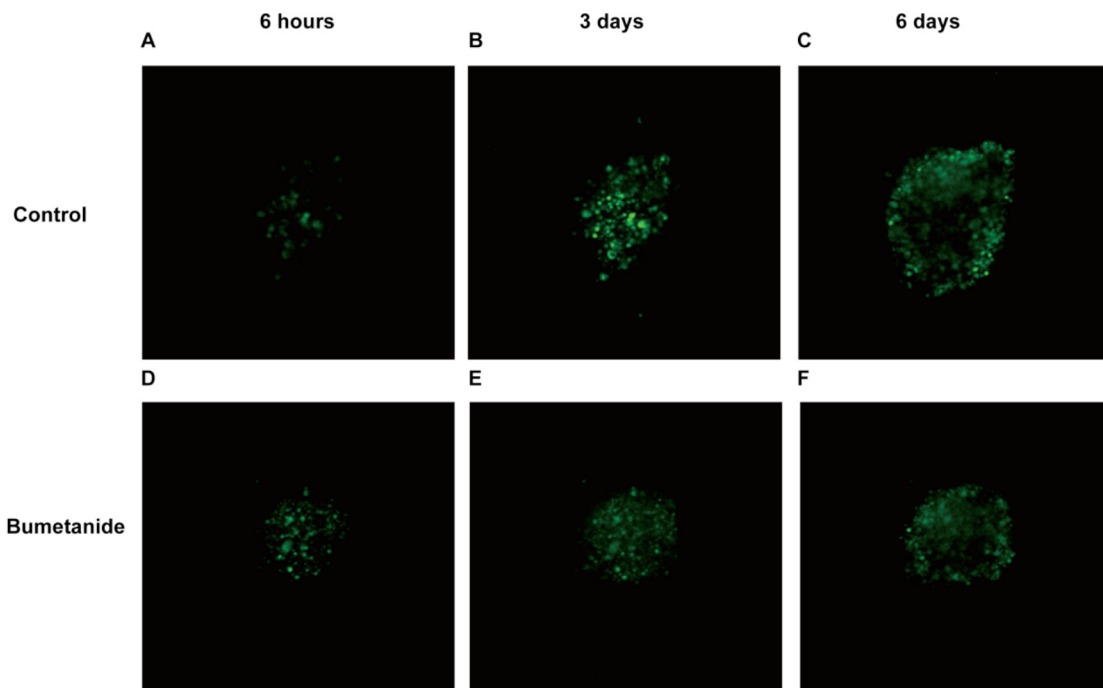


Figure 4. Glioma cell invasion in brain slice “co-cultures”. The EGFP-expressing U251MG cell line-derived spheroids were implanted into 3 days cultured brain slice close to the corpus callosum. Confocal fluorescence micrographs show invasion of glioma cells after cocultured for 6 hours, 3 days and 6 days. **A-C**, In the control group treated with PBS, the spheroid area and density increased apparently over time. **D-F**, In the “co-culture” system treated with bumetanide, the spheroid area and density increased slightly.

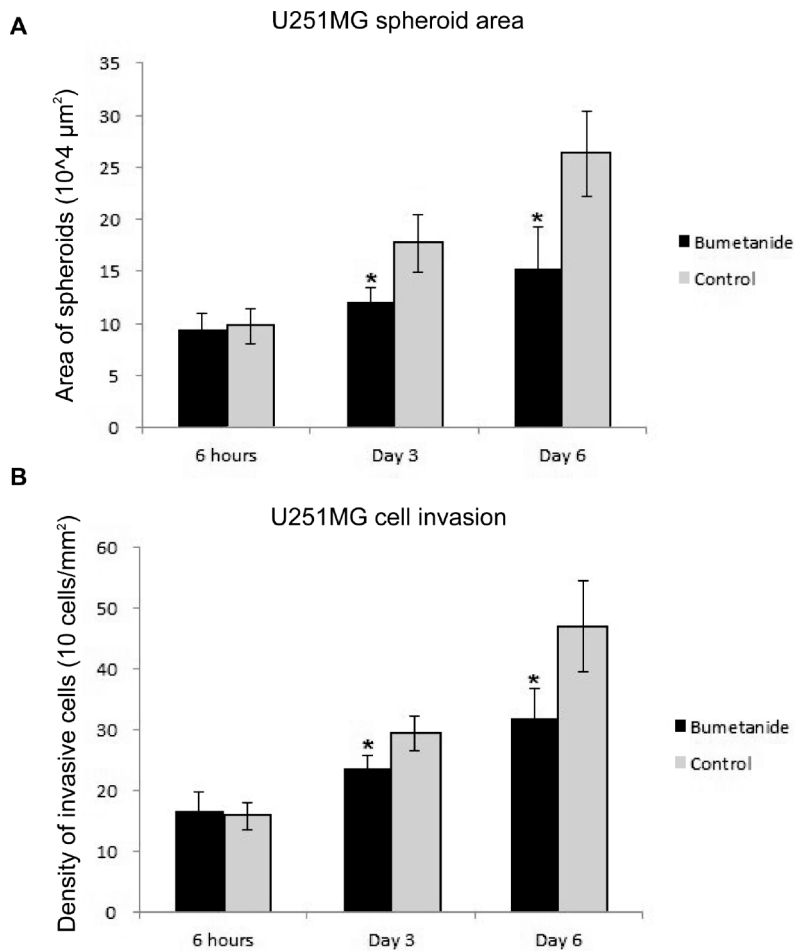


Figure 5. Confocal fluorescence micrographs with imposed Z-stack images of U251MG spheroids show the spheroid area (**A**) and cell invasion (**B**) and values were obtained at 6 hours, day 3 and day 6 after implantation, respectively. Values represent means \pm SD of three independent experiments ($p < 0.05$ compared with the control group).

confocal laser microscopy. Compared with other similar invasion studies, the tumor cell invasion has been investigated at the end of the experiments, and not at different time points during the process of invasion in the same individual “co-cultures”^{29,30}.

Using brain slice model, we investigate the anti-glioma cell invasion by applying a NKCC1 inhibitor, bumetanide, which is a diuretic agent. As a transporter belongs to the SLC12A family of cation-chloride cotransporters, NKCC1 is a fundamental transporter utilized in the regulation of intracellular volume and the accumulation of intracellular Cl⁻³¹. Also, NKCC1 is regarded as a factor which can promote glioma cell invasion^{32,33}. We demonstrate that inhibition of NKCC1 by bumetanide can prevent glioma cell invasion with brain slice “co-culture” for the first time. This procedure can highly simulate the natural process of glioma invasion within the normal brain. Meanwhile, NKCC1 is involved in glioma migration through regulation of focal adhesion and cell contractility^{13,14}. Activation of NKCC1 is regulat-

ed by a family of kinases named the With-No-K (Lysine) kinases (WNKs, WNK1-4)^{34,35}.

Conclusions

In this study, we prove that the brain slice model maintains the normal brain morphology and connectivity which is significant to tumor invasion model. Also, the brain slice “co-culture” can be facilitated to observe glioma migration and invasion. In particular, the “co-culture” system strives to combine the versatility of tumor micro-environment, invasion assays with complexity of the organotypic situation. In conclusion, this organotypic brain slice “co-culture” model seems to be an ideal model suitable for drug development and glioma *in vitro* assays.

Conflicts of interest

The authors declare no conflicts of interest.

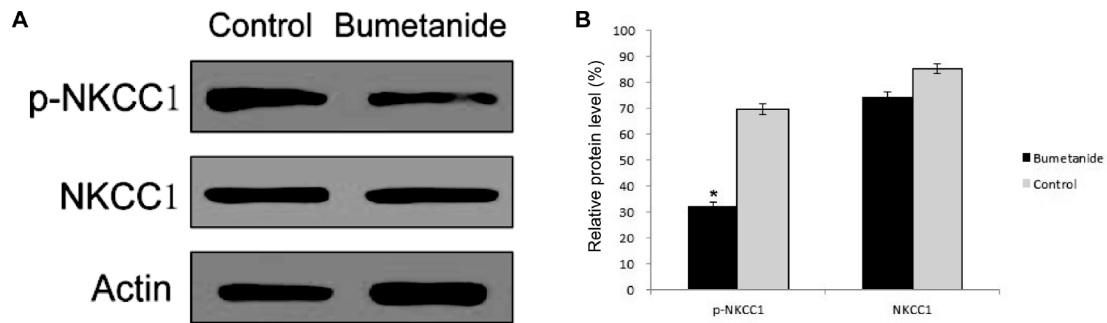


Figure 6. p-NKCC1 expression of glioma cells. A Bands of the western blot in control group and bumetanide-treated group. B The expression level of p-NKCC1 was apparently lower in the bumetanide-treated group compared to the control group. Values represent means \pm SD of 3 independent experiments. (* $p < 0.05$ compared with the control group).

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