Acid-sensing ion channel 1 (ASIC1) mediates weak acid-induced migration of human malignant glioma cells

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Abstract: Glioblastoma is the most aggressive and lethal tumor in the central nervous system in adult and has poor prognosis due to strong proliferation and aggressive invasion capacity. Acidic microenvironment is commonly observed in tumor tissues but the exact role of acidosis in the pathophysiology of glioblastoma and underlying mechanisms remain unclear. Acid-sensing ion channels (ASICs) are proton-gated cation channels activated by low extracellular pH. Recent studies have suggested that ASICs are involved in the pathogenesis of some tumors, such as lung cancer and breast cancer. But the effect of acidosis and activation of ASICs on malignant glioma of the central nervous system has not been reported. In this study, we investigated the expression of ASIC1 in human glioma cell lines (U87MG and A172) and its possible effect on the proliferation and migration of these cells. The results demonstrated that ASIC1 is functionally expressed in U87MG and A172 cells. Treatment with extracellular weak acid (pH 7.0) has no effect on the proliferation but increases the migration of the two cell lines. Application of PcTX1, a specific inhibitor of ASIC1a and ASIC1a/2b channels, or knocking down ASIC1 by siRNA, can abolish the effect of weak acid-induced cell migration. Together, our results indicate that ASIC1 mediates extracellular weak acid induced migration of human malignant glioma cells and may therefore serve as a therapeutic target for malignant glioma in human.

Keywords: Acid-sensing ion channels, malignant glioma, acidic microenvironment, weak acid, proliferation, migration

Introduction

Glioblastoma is a broad category of tumors derived from glial cells that support nerve cells in the brain and spinal cord. Every year, about 100,000 people worldwide are diagnosed with glioblastoma multiforme, a malignant glioma [1]. Although it represents only one to two percent of all newly diagnosed cancers, glioblastoma is one of the most disabling and lethal diseases [2]. The treatment and prognosis of glioblastoma depend on a series of factors, such as age, tumor type, and location of the tumor in the brain. The recurrence and poor prognosis of malignant glioma are mainly due to its strong ability of uncontrolled proliferation and aggressive invasion [3]. The infiltrative growth makes surgical removal very difficult, sometimes impossible. In addition, the high resistance of malignant glioma cells to therapies and the vulnerability of the central nervous system hamper the antitumor interventions [4]. It is hoped that potential new mechanisms and targets can be discovered for effective therapies to prolong the survival of glioblastoma patients.

Acidosis is one of the most common changes of extracellular microenvironment in tumors [5, 6]. The pH of normal tissues is kept within tight limits under physiological conditions. In pathological situations, such as during tumor growth, ischemia and inflammation, however, local pH drop and long-lasting acidification develop. An acidosis often occurs in tumors with high metabolic rates owing to blood hypoperfusion, local hypoxia, and increased glycolytic activity [7]. The acidic microenvironment is harmful to most normal cells, but it is often favorable to solid tumor cells although the precise mechanisms remain elusive [7-9].
Acid-sensing ion channels (ASICs) are voltage-insensitive cationic channels activated by extracellular acidification. ASICs form a subfamily of the epithelial sodium channels/degenerin (ENaC/Deg) family. Four different genes encode six ASIC subunits: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4. They can form functional hetero/homotrimeric channels that are activated by acidic pH and inhibited by amiloride, a commonly used non-selective ASIC antagonist [10]. ASICs are expressed with high levels in the nervous system, which are responsible for the acid-evoked currents and act as pH sensors leading to excitation in neurons [11]. Evidence has shown that ASICs play physiological and pathological roles in many processes, such as pain sensation, learning, fear behavior, and neurodegeneration after ischemic stroke [9]. The expression of ASICs in high grade glioma cells has been reported over a decade ago [12], however, the exact role of these channels in the pathogenesis of glioma remains elusive. Although accumulating evidence showed that acidic conditions can affect tumor progression by promoting cell migration, metastasis, invasion and angiogenesis [13-16], the potential mechanism is not well known. In the present study, we demonstrated that weak acid (pH 7.0) promotes the migration of malignant glioma cells, at least in part, by activating ASIC1 channels.

Materials and methods

Reagents and antibodies

Polyclonal anti-ASIC1 antibody for Western blot was a gift from Dr. Xiangming Zha (University of South Alabama College of Medicine, Mobile, Alabama) [17]. Vybrant® MTT cell proliferation assay kit (V-13154) used for cell viability assay was purchased from Thermo Fisher Scientific (Rockford, USA). SiRNA targeting the human ASIC1 (Product#: NM_001095, siRNA ID: SASI-Hs01_00167899) and SiRNA negative control (SIC001) were synthesized and purified by Sigma-Aldrich (St. Louis, Mo).

Cell culture and transfection

The human glioblastoma cell lines U87MG and A172 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured with Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 μg/ml streptomycin (PS) at 37°C in humidified atmosphere incubator containing 5% CO₂. SiRNAs (targeting the human ASIC1 and negative control) were transfected using Lipofectamine® RNAiMAX reagent (Invitrogen, Carlsbad, CA) in serum free DMEM medium at a final concentration of 10 nM according to the manufacturer’s instructions. After 24 hours of transfection, cells were further grown for 24 hours in growth medium before Western blotting, wound-healing and transwell migration assays.

Western blotting

Cells were lysed using M-PER Mammalian Protein Extraction Reagent with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, USA). Samples were then centrifuged at 13200 g for 20 minutes at 4°C, protein concentrations were measured using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant of lysates was transferred to a new tube with 4 x loading buffer and boiled for 10 minutes. All the samples were separated by 10% SDS-PAGE gel, and then transferred onto PVDF membranes (Millipore, Germany). Primary antibodies were diluted at the following ratios: rabbit anti-ASIC1 (1:1000), and rabbit anti-β-actin (1:8000). Blots were incubated with primary antibodies overnight at 4°C followed by the HRP-conjugated secondary antibodies (Thermo Fisher Scientific, 1:5000) for 1 hour. The signals were identified using an ECL kit (Millipore, Germany) and the band images were acquired using Image Quant LAS 4000.

Whole-cell patch-clamp recording

Whole-cell currents were recorded by patch-clamp techniques as described in our previous study [18]. The currents were recorded with an Axopatch 200B amplifier and low-pass filtered at 2 KHz, digitized using Digidata 1320 DAC unit. Cells were clamped at a holding potential of -80 mV. The patch pipettes were made from thin walled borosilicate glass micropipettes (1.5 mm diameter, World Precision Instruments, Sarasota, FL) by a micropipette puller (PP83; Tokyo, Japan). The range of pipette resistance was 3-5 MΩ when filled with intracellular solution contained 140 mM CsF, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 11 mM EGTA, 2 mM tetraethylammonium chloride, and 4 mM MgATP; pH 7.3 adjusted with CsOH, 290 to 300 mOsm
adjusted with sucrose. Cells were perfused with an extracellular fluid (ECF) contained 140 mM NaCl, 5.4 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM Glucose, and 10 mM HEPES; pH 7.4 and 6.0 adjusted with NaOH, 320-330 mOsm. PcTX1 (Peptide International) was dissolved in ddH$_2$O at 20 μM stock solution before adding to ECF. The data were recorded and analyzed with pClamp and Sigma Plot software.

**Cell viability assay**

Cells were seeded in 96-well plates overnight and then incubated with pH 7.4 or 7.0 ECF in the absence or presence of 20 nM PcTX1 for 3 h, and then changed to culture medium containing 5% FBS incubating for 24 h, 48 h and 72 h. After the treatments, cell viability was measured by MTT Cell Proliferation Assay Kit according to the manufacturer’s instructions. The absorbance values at 540 nm were detected by spectrometer (Molecular devices).

**Wound-healing assay**

Wound-healing assay was performed as described in our previous studies [19]. Cells were seeded into a 24-well plate and grown to ~80% confluence. The cell monolayer was scratched with 200 μl sterile pipette tip and treated with ECF with or without PcTX1 for 3 hours. The original wound areas at 0 hour were photographed with microscope (Olympus FSX100, 4 ×). After 3 hours, cells were then incubated with culture medium containing 1% FBS for 24 hours. 24 hours later, images were taken from the same location of the original wound areas and the migration distance was measured with Image J software. The wound-healing rate was calculated as: the ratio of wound closure = [(Area of original wound at 0 h - Area of wound after healing at 24 h)/Area of original wound at 0 h] × 100%.

**Transwell migration assay**

Transwell assay was performed as described in our previous studies [19]. The assay is based on two compartments separated by a membrane with an accurately defined pore size. Cell transwell chambers with 8-μm pore size of polycarbonate membrane filters (Corning, USA) were first pre-coated with poly-L-ornithine overnight. Before plating cells, chambers were incubated with serum-free culture medium for 30 minutes. After that, cells were seeded with culture medium containing 1% FBS in the upper-chambers that were immersed to 750 μl whole medium (DMEM with 10% FBS). After incubating for another 24 h, some cells on the upper surface of the filter migrated through the membrane to the lower surface of the filter. Cells on the upper face of the filter were wiped out thoroughly with cotton swabs, and those attached on the lower surface were fixed with 4% paraformaldehyde for 15 min. They were then stained with 0.5% crystal violet for 10 min, and let the membrane air dry. The cells on the lower surface of the membrane filter were counted under a microscope in 3-4 random fields with 10 × magnification. Cell numbers in each group were normalized to the control groups. Assays were performed in at least 3 independent experiments.

**Statistical analysis**

The statistical significance was analyzed using the Prism 8.0.1 software (GraphPad Software, San Diego, CA, USA). The data were expressed as mean ± SEM. Groups were compared using one-way ANOVA followed by Tukey’s multiple comparison test or unpaired Student’s t test where appropriate. $P < 0.05$ was regarded as statistically significant.

**Results**

**Functional ASIC1 is expressed in human glioma cells**

To determine whether ASIC1 is expressed in human glioma cells, we first investigated the expression of ASIC1 protein in A172 and U87-MG cells by Western blotting. Results showed that both of the two glioblastoma cell lines express ASIC1 protein, especially the U87MG cells (Figure 1A). Next, we detected the existence of acid-activated currents and the effect of pharmacologic agents on the currents. Acid-activated currents were recorded by whole cell patch-clamp as described in our previous studies [18]. As shown in Figure 1B, drop of extracellular pH from 7.4 to 6.0 can induce transient inward currents in A172 and U87MG cells. PcTX1 from venom of tarantula Psalmopoeus cambridgei is a specific blocker for homomeric ASIC1a and heteromeric ASIC1a/ASIC2b channels [20], which potently inhibits the proton-gated currents mediated by ASIC1a. For this reason, we confirmed the effect of PcTX1 on acid-activated currents. As shown in Figure 1C and 1D, the peak of acid-activated currents...
was decreased by 80% after several minutes of PcTX1 (20 nM) perfusion (***P < 0.001, unpaired student’s t test, n=5) indicating that the majority of currents activated by extracellular acid are mediated by ASIC1.

**Weak acid has no effect on proliferation of human glioma cells**

Previous study showed that severe acidosis (e.g. pH 6.0) promotes the proliferation of some type of glioma cells [21]. In this study, we first tested the effect of weak acid on the proliferation of A172 and U87MG cells. We used either pH 7.4 or pH 7.0 solution to pretreat cells for 3 h, and then changed back to medium containing 5% FBS. The cell viability was measured at 24 h, 48 h and 72 h after acid treatment by MTT assay. Results showed that treatment of cells with pH 7.0 solution had no significant effect on the proliferation of A172 cells at 24 h, 48 h and 72 h, and PcTX1 had no influence on the proliferation of A172 cells (one-way ANOVA, n=3, Figure 2A and 2B). Similarly, treatment with weak acid (pH 7.0) and PcTX1 had no clear effect on the proliferation of U87MG cells at different time points (one-way ANOVA, n=3, Figure 2C and 2D).

**Weak acid promotes the migration of human glioma cells in transwell assay**

To further examine the role of ASIC1 in glioma cell migration, we performed a transwell migration assay using inserts with an 8-μm pore size. The glioma cells were cultured in the upper chambers for 24 h, and then pretreated with pH 7.0 in the absence or presence of PcTX1 for 3 h. After 24 h, the number of migrated cells in the lower chamber was examined. Compared with pH 7.4, weak acid (pH 7.0) greatly increased the number of migrated A172 cells by 37%, and PcTX1 (20 nM) completely inhibited the acid-induced migration of A172 cells by 37%, and PcTX1 (20 nM) completely inhibited the acid-induced migration of A172 cells (*P < 0.05, **P < 0.01, one-way ANOVA, n=4). Similarly, weak acid (pH 7.0) stimulated the migration of U87MG cells by 67% and PcTX1 (20 nM) blocked the acid-induced increase in migration of these cells (*P < 0.05, **P < 0.01, one-way ANOVA, n=3, Figure 4C and 4D).

**Knockdown of ASIC1 inhibits migration of glioma cells**

Next, we used small interference RNA to silence ASIC1 expression and further determined the...
ASIC1 and migration of malignant glioma cells

A

![Images showing cell morphology under different pH conditions](image)

B

![Graphs showing optical density values under different conditions](image)

C

![Images showing cell morphology under different pH conditions](image)

D

![Graphs showing optical density values under different conditions](image)
ASIC1 and migration of malignant glioma cells

Figure 2. Effects of acidosis and PcTX1 on proliferation of A172 and U87MG cells. (A) Representative micrographs of 24 h and 72 h and (B) statistical results of 24 h, 48 h and 72 h showing the presence or lack of effects of extracellular weak acid pH 7.0 and PcTX1 on the proliferation of A172 cells. (C) Representative micrographs of 24 h and 72 h and (D) statistical results of 24 h, 48 h and 72 h showing the presence or lack of effect of extracellular weak acid and PcTX1 on the proliferation of U87MG cells. Data are expressed as mean ± SEM. n=3. Scale bar =100 µm. One-way ANOVA followed by Tukey’s multiple comparison test.

Figure 3. Effects of extracellular acidosis and PcTX1 on migration of A172 and U87MG cells by wound healing. (A) Representative micrographs and (B) statistical results showing the effect of extracellular weak acid and PcTX1 on migration of A172 cells by wound-healing assay. (C) Representative micrographs and (D) statistical results showing
the effect of extracellular acid and PcTX1 on the migration of U87MG cells by wound-healing assay. Data are expressed as mean ± SEM. n=3-4. Scale bar =250 µm. *P < 0.05, **P < 0.01, by one-way ANOVA followed by Tukey’s multiple comparison test.

Figure 4. Effects of extracellular acidosis and PcTX1 on migration of A172 and U87MG cells by transwell. (A) Representative micrographs and (B) statistical results showing the effects of extracellular acid and PcTX1 on the migration of A172 cells by transwell assay. (C) Representative micrographs and (D) statistical results showing the effects of extracellular acid and PcTX1 on the migration of U87MG cells by transwell assay. Data are expressed as mean ± SEM. n=4-5. Scale bar =100 µm. *P < 0.05, by one-way ANOVA followed by Tukey’s multiple comparison test.

influence of ASIC1 protein on the migration of glioma cells under acidic condition. Owing to the low ASIC1 expression in A172 cells, we used U87MG in this experiment to test the effect of ASIC1 siRNA. As shown in Figure 5A, siRNA-ASIC1 decreased the expression of
ASIC1 and migration of malignant glioma cells

A

B

C

D

E

F

G

H
ASIC1 protein expression in U87MG cells by 72% when compared with siRNA-Control (***P < 0.001, unpaired student’s t test, n=4, Figure 5A and 5B). Next, we examined whether knockdown of ASIC1 affects cell proliferation in the presence or absence of extracellular weak acid. MTT assay was performed to detect the cell viability at 24 h and 72 h with and without acid treatment both in siRNA-Control and siRNA-ASIC1 groups. Results showed that knockdown of ASIC1 had no obvious effect on the proliferation of U87MG cells both at 24 h and 72 h either without or with the treatment of extracellular weak acid (one-way ANOVA, n=3, Figure 5C and 5D). We further examined whether knockdown of ASIC1 has an effect on the migration of U87MG cells. As shown in wound healing assay, ASIC1 knockdown did not have an effect on the migration under normal pH (7.4), but it reduced the migration by 44% under acidic condition (pH 7.0) when compared with siRNA-Control (*P < 0.05, one-way ANOVA, n=5, Figure 5E and 5F). The effect of ASIC1 knockdown on migration was also assessed by transwell. As shown in Figure 5G and 5H, weak acid (pH 7.0) produced a large increase in the migration of siRNA-Control treated U87MG cells, however, it had no effect on the migration of siRNA-ASIC1 treated cells (*P < 0.05, ***P < 0.001, one-way ANOVA, n=4). These results further suggested that ASIC1 is involved in weak acid-induced migration of malignant human glioma cells.

Discussion

Glioblastoma multiforme, with a high rate of morbidity and mortality, is the most aggressive type of cancer in central nervous system (CNS), and accounts for approximately 15% of all brain tumors and 60-75% of astrocytic tumors [22, 23]. Due to its uncontrollable infiltrative nature and resistant to radiation and chemotherapy, the treatment effect of conventional therapies, such as radiotherapy, chemotherapy or surgery, is seriously hindered for glioma, making it one of the most serious solid tumors threatening patients’ life [24, 25]. Therefore, it is of urgent need to understand the complex interaction between glioma cells and their microenvironment, identify the potential molecular mechanisms underlying their aggressive behaviors, and explore new therapeutic strategies to control them. Our present study demonstrated that ASICs were expressed in U87MG and A172 glioma cell lines and enhanced the migration capacity of these cells induced by weak acidic stimulation. Furthermore, we found that application of a commonly used ASIC1a and ASIC1a/2b channel inhibitor, PpTX1, or inducing ASIC1 knockdown strongly inhibited acid-mediated migration of U87MG cells. Taken together, our results suggest that ASIC1 mediates extracellular weak acid induced migration of malignant glioma cells.

Glioblastoma is hard to be treated owing to the complex microenvironment. Due to high metabolic rate, all the tumor microenvironment in humans and animal models is both hypoxic [26, 27] and acidic [28-31]. Much work has been performed on the effect of low pH upon normal cells, and low pH microenvironment has been shown to inhibit cell proliferation, activity and survival [32]. However, different from normal cells, the tumor environment is normally acidic, especially in solid tumors [33, 34]. Solid tumors grow rapidly, to maintain the sufficient supply of oxygen and nutrients, tumor cells tend to switch energy metabolism toward glycolysis [35, 36]. The high endogenous metabolism rate and enhanced glycolysis of tumor cells cause the accumulation of lactic acid and carbon dioxide, leading to acidic microenvironment, with a pH as low as 5.8 [28, 31]. The microenvironment plays crucial role in tumor progression, such as increased mutations [37], resistance to apoptosis [38] and enhanced invasion and metastasis [39, 40]. In this study, we found that extracellular weak acid (e.g. pH 7.0) promoted the

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**Figure 5.** Silencing ASIC1 inhibits the migration of U87MG cells. (A) Representative Western blot bands and (B) quantification of the ASIC1 expression showing the transfection efficiency of siRNA-ASIC1 in U87MG cells. (C) Representative micrographs and (D) statistical results showing that knockdown of ASIC1 has no obvious effect on the proliferation of U87MG cells both at 24 h and 72 h without or with the treatment of extracellular weak acid. (E) Representative micrographs and (F) statistical results showing the effect of siRNA-Control or siRNA-ASIC1 on acid-induced migration of U87MG cells by wound-healing assay. (G) Representative micrographs and (H) statistical results showing the effects of siRNA-Control or siRNA-ASIC1 on acid-induced migration of U87MG cells by transwell assay. Data are expressed as mean ± SEM. n=3-5. Black bar =250 μm. White bar =100 μm. *P < 0.05, **P < 0.01, ***P < 0.001, by one-way ANOVA followed by Tukey’s multiple comparison test.
migration of U87MG and A172 glioma cells. We have also tried lower pH, e.g. pH 6.5, but it clearly affected the viability of U87MG and A172 cells (data not shown), making it difficult to study the migration property of these cells.

The poor prognosis of glioma is mainly due to the powerful ability of cellular proliferation and invasion [3]. Previous studies have shown that ASICs activation mediated by extracellular acidosis affects proliferation or migration in some tumor cells such as mammary carcinoma and lung cancer cells [12, 21, 34], but there are no studies on malignant glioma of the central nervous system. We provided both biochemical and electrophysiological evidence suggesting the expression of functional ASICs in two distinct human glioblastoma cell lines. We detected the ASIC1 protein expression and recorded ASIC currents in U87MG and A172 cell lines. We found that the amplitude of inward current of ASIC in A172 was lower than U87MG cells, which was probably consistent with the lower expression of ASIC1 protein in A172 cells. Further, the inward currents were blocked by PcTX1. The electrophysiological and pharmacological characterizations revealed clear evidence for the presence of functional ASIC1 channels in U87MG cells. Our results first showed that weak acid (pH 7.0) incubation had no effects on the proliferation, but clearly enhanced the migration of malignant human glioma cells (U87MG and A172).

The most aggressive tumor, glioblastoma multiforme, hampered the treatment efficacy due to the lack of control on glioma cell growth and differentiation, and the lack of detailed knowledge of how the transformed phenotype evolves and how the tumor adapts to the microenvironment to support metabolic demand, etc. [12]. It has been shown that amiloride-sensitive inward Na+ channel is constitutively activated in glioma cells, but not in normal and low grade astrocytes [41]. Additionally, the up-regulation of Cl− and K+ channels in glioma cells is not found in normal glia, at least functionally [42, 43]. Therefore, it is reasonable to assume that the specific ion transport system is closely related to the characteristics of malignant gliomas, such as significant growth and migration ability. We then explored the effect of ASIC1 activation on glioma proliferation and migration. As shown in our results, weak acid (pH 7.0) had no effect on the proliferation of A172 and U87MG cells measured both at 24 h and 72 h, but it apparently increased their capacity of migration as shown by wound-healing and transwell assays. Application of ASIC1 inhibitor, PcTX1, effectively blocked the acid-induced migration of the two cells, suggesting that weak acid stimulated glioma migration is mediated, at least in part, by ASIC1 activation. To further determine the role of ASIC1 in the migration of glioma cells, we also examined the effect of knocking down ASIC1 on glioma migration. We found that specific siRNA-ASIC1 down-regulated the expression of ASIC1 in U87MG cells and knocking down the ASIC1 channel impaired the migration ability of these cells.

Ion channels are considered to be crucial in the invasive capacity of tumor cells and formation of metastases [44]. Previous researches showed that voltage-gated K+ channels, such as K1,3, K10.1, and K11.1, play a role in cell migration [45]. In addition, voltage-gated Na+ channels are expressed in metastatic tumor cells and had positive impact on cell migration and invasion [46]. Besides K+ and Na+, Ca2+ is also important for cancer cell migration [34]. As we all know, the extracellular microenvironment of tumor tissues is often acidic [47]. Extracellular pH changes are known to have impact on the activity of a variety of membrane receptors and ion channels. Acid-sensing ion channels (ASICs) are H+ -gated cation channels activated by extracellular acid. The high H+ sensitivity makes them attractive signaling molecules to adapt the tumor behavior to acidic microenvironment. Recent studies have shown that acidic microenvironment activates ASICs and induces ROS generation, contributing to the pathogenesis of breast cancer [34]. ASIC-calcium signaling also plays a role in the effect of acidic microenvironment on proliferation and migration of lung cancer cells [21]. In our present study, we showed that the activation of ASIC1 mediates extracellular weak acid induced migration of malignant human glioma cells.

In summary, our study displayed the first evidence that supports a significant role of this channel in the pathogenesis of human gliomas.

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Disclosure of conflict of interest

None.

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