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Research Paper

NKCC1 Regulates Migration Ability of Glioblastoma Cells by Modulation of Actin Dynamics and Interacting with Cofilin

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ABSTRACT

Glioblastoma (GBM) is the most aggressive primary brain tumor in adults. The mechanisms that confer GBM cells their invasive behavior are poorly understood. The electroneutral Na^+-K^+-$2Cl^-$ co-transporter 1 (NKCC1) is an important cell volume regulator that participates in cell migration. We have shown that inhibition of NKCC1 in GBM cells leads to decreased cell migration, *in vitro* and *in vivo*. We now report on the role of NKCC1 on cytoskeletal dynamics. We show that GBM cells display a significant decrease in F-actin content upon NKCC1 knockdown (NKCC1-KD). To determine the potential actin-regulatory mechanisms affected by NKCC1 inhibition, we studied NKCC1 protein interactions. We found that NKCC1 interacts with the actin-regulating protein Cofilin-1 and can regulate its membrane localization. Finally, we analyzed whether NKCC1 could regulate the activity of the small Rho-GTPases RhoA and Rac1. We observed that the active forms of RhoA and Rac1 were decreased in NKCC1-KD cells. In summary, we report that NKCC1 regulates GBM cell migration by modulating the cytoskeleton through multiple targets including F-actin regulation through Cofilin-1 and RhoGTPase activity. Due to its essential role in cell migration NKCC1 may serve as a specific therapeutic target to decrease cell invasion in patients with primary brain cancer.

1. Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor of the Central Nervous System (Siegel et al., 2013; Chaichana et al., 2011; Chaichana et al., 2014). GBM has cellular heterogeneity and displays key features of invasion and infiltration of healthy brain tissue (Quinones-Hinojosa and Chaichana, 2007; Giese et al., 2003). Despite multimodal therapy including surgery, radiation, and chemotherapy, the median survival is 14.6 months, and the prognosis remains dismal due to tumor recurrence (Stupp et al., 2005; Chaichana et al., 2014). This high rate of recurrence can be attributed to the high migration capacity of GBM cells as well as brain tumor initiating cells that are resistant to treatment (Guerrero-Cazares et al., 2012; Lathia et al., 2015). For these reasons, targeting proteins that promote cell migration may potentially result in better therapeutic strategies.

During cell migration, GBM cells require modifying their cellular volume to go through narrow spaces; these changes in volume are regulated through ionic transport (Watkins and Sontheimer, 2011). Ion co-transporters, such as the Sodium Potassium Chloride co-transporter (NKCC1) 1, regulate intracellular volume and Cl_2^- accumulation, allowing the movement of Na^+, K^+ and Cl^- ions across the plasma membrane using the energy generated by the Na^+/K^+ ATPase.

Previously, we and others have determined that NKCC1 inhibition decreases GBM cell migration and invasion *in vitro* and *in vivo* (Garzon-Muvdi et al., 2012; Haas and Sontheimer, 2010). In addition, we found that NKCC1 expression levels are increased in GBM tissues (with respect to normal cortex) and that NKCC1 modulates glioma cell invasion through the regulation of cell contractility and focal adhesion dynamics (Garzon-Muvdi et al., 2012). Furthermore, we found that EGF stimulation increases the presence of active (*i.e.* phosphorylated) NKCC1 (Garzon-Muvdi et al., 2012). However, the intracellular mechanisms utilized by NKCC1 to regulate cell migration and adhesion changes have not been elucidated. Cytoskeleton dynamics have the potential of being the converging phenomenon that links NKCC1 activity, cell migration, and cell adhesion.

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Cell movement is driven by the assembling of actin filaments at the leading edge of the cell, providing a major force to drive cell protrusions, changes in shape, migration and invasion (Insall and Machesky, 2009; Pollard and Borisy, 2003). One of the key regulators of actin assembly is Cofilin 1, which is involved in determining the direction of the protrusion and promotes lamellipodium extension and cell migration (Chen et al., 2001). Cofilin 1 severs actin filaments to produce free actin barbed ends, required for new actin polymerization (Desmarais et al., 2005; Chan et al., 2000). Actin dynamics regulated by Cofilin 1 are coupled with the activation of Rho and Rac1 family of GTPases, which are key intermediates in signal transduction driving cytoskeleton organization (Lauffenburger and Horwitz, 1996; Lang et al., 1998; Fortin Ensign et al., 2013; Nakada et al., 2007; Kwiatkowska and Symons, 2013). Interestingly, Cofilin 1 has been implicated in promoting metastasis and invasion in breast and prostate cancer, allowing the formation of filopodia and enhancing migration activity (Bravo-Cordero et al., 2013; Sidani et al., 2007).

Here, we report that NKCC1 regulates the actin cytoskeleton in primary patient-derived GBM cells serving as a protein scaffold to Cofilin, thus facilitating its localization at the plasma membrane. Upon NKCC1 knockdown, there is a decreased expression of Cofilin1 at the plasma membrane coupled with a decrease of RhoA and Rac1 activity. These events lead to a reduction in the formation of filamentous actin, delayed cell spreading, and reduced migration. Our data shows NKCC1 as a potential component of the actin cytoskeleton machinery of primary-derived GBM cells. Our results suggest that targeting NKCC1 in GBM will decrease cell dispersal by disrupting cytoskeleton dynamics.

2. Materials and Methods

2.1. Cell Lines

Patient samples of glioma tissues were obtained at the Johns Hopkins Hospital under the approval of the Institutional Review Board (IRB). All human brain tumor cell lines were derived from intraoperative tissue samples from patients treated surgically for newly diagnosed glioblastoma without prior treatment. Clinical data for primary GBM cell lines 318, 612 and 965 is described in detail in Table S1. Additionally, we used Human Embryonic Kidney 293 (HEK293) and MCF10A (mammary gland/breast derived cells) cells which were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured according to manufacturer instructions. Primary GBM cell lines 318, 612 and 965 have been analyzed by our group previously (Garzon-Muvdi et al., 2012; Yang et al., 2017; Smith et al., 2016) and were cultured using Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, B27 serum free supplement (Gibco), 20 ng/mL epidermal growth factor (EGF), and 20 ng/mL fibroblast-derived growth factor (FGF). HEK293 and MCF10A were cultured according to manufacturer instructions.

2.2. Viral Transduction

We used a human clone set (Sigma Aldrich Mission) of sequence verified lentiviral particles (pLKO.1, TRC0000296498) that target human NKCC1 (SLC12A2) and TRC2-pLKO-puro empty vector control (ref: SHC201) to generate NKCC1-shRNA stably expressing cell lines. Seventy-two hours after transduction, cells were cultured in the presence of puromycin to select cells with successful transduction. Knockdown of NKCC1 was confirmed by immunoblot before each experiment. In addition, the F-actin biosensor Lifeact (Riedl et al., 2008) cloned into lentiviral particles (kindly provided by Peter Devreotes laboratory) was used to transduce GBM cells. Throughout the text empty vector control cells are referred as EV and NKCC1-shRNA cells are referred as NKCC1-KD.

2.3. Cloning of Full-length Human NKCC1 and Generation of EGFP Fusion Protein

As previously described by our group (Garzon-Muvdi et al., 2012) we have cloned the human NKCC1 protein into a PCDNA3-EGFP expression vector (NKCC1-GFP plasmids).

2.4. Immunoblotting

Cells were plated on a 25 cm^2 flask or 6 well plates and exposed to the different experimental conditions. Cell were harvested using RIPA lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1% NP40, 1% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche)), and HaltTM Phosphatase inhibitor cocktail (Thermo). Proteins from whole cell lysates were resolved using the NuPAGE 4–12% Bis-Tris gradient gel (Invitrogen). The Subcellular Protein Fractionation Kit was used for membrane and cytosol protein extraction (Thermo Scientific). Proteins were transferred to PVDF membrane, blocked in 5% non-fat milk or 2% bovine serum albumin in TBS-Tween-20, and probed with the antibodies for NKCC1 (ref: 8351, Cell signaling), MLC2 (ref: 3672, Cell signaling), pMLC2 (ref: 3671, Cell signaling), Cofilin1 (ref:5175, Cell signaling), pCofilin (ref: 3311, Cell signaling, Ser3); GFP (ref:632459, Clontech), Rac1 (ref; sc-95, Santa cruz), RhoA (ref: sc-418, Santa Cruz), GAPDH (ref: sc-32233, Santa Cruz), EGFR (ref: 06-847, Millipore), pEGFR (ref: 2234, Cell Signaling) and Actin (ref: ab8227, AbCAM).

MCF10A cells were used to test pMLC2 and tMLC2 phosphorylation due to a high signal-to-noise ratio using the GBM primary-derived cells. Detection was performed with the appropriate horseradish-peroxidase conjugated secondary antibodies and using enhanced chemiluminescence reagent (GE Healthcare Life Science). Densitometry analysis was performed using the Gel Analysis tool from ImageJ.

2.5. Assessment of Migration on Nano-patterned Groove Surface

Migration of glioma cells was quantified using a directional migration assay using nano-ridges/grooves constructed of transparent poly (urethane acrylate) (PUA) as previously described (Garzon-Muvdi et al., 2012; Smith et al., 2016; Kondapalli et al., 2015; Tilghman et al., 2016). Nanopattern surfaces were coated with laminin (3 $\mu g/cm^2$). Cell migration was quantified using timelapse microscopy. Long-term observation was done on a motorized inverted microscope (Olympus IX81) equipped with a Cascade 512B II CCD camera and temperature and gas controlling environmental chamber. Phase-contrast and epifluorescent cell images were automatically recorded under 10× objective (NA = 0.30) using the Slidebook 4.1 (Intelligent Imaging Innovations, Denver, CO) for 15 h at 10–20 min intervals. We performed time-lapse videomicroscopy in order to measure cell migration speed, distance, and directional persistence. The obtained images were analyzed using Matlab with a previously written cell tracking script developed in our laboratory (Garzon-Muvdi et al., 2012). Videos consisted of 60 timeframes reflecting a total time of 20 min. Cells were selected manually for tracking and cells were discarded from analysis if they went into apoptosis, mitosis or migrated out of view.

2.6. Immunoprecipitation

For the measuring RhoGTPase activity we performed a pulldown assay of Rac1-GTP, RhoA-GTP using the RhoA/Rac1/Cdc42 Activation Assay Kit (Cell Biolabs, Inc.). The methodology used was followed according to the manufacturer's instructions. Briefly, the adherent cultured cells were lysed, centrifuged and the supernatant was collected for further steps. PAK PBD Agarose beads were added to an aliquot of the supernatant. After incubation, the beads were pelleted by centrifugation. The supernatant was discarded and the bead pellet was washed thrice with the supplied assay buffer, centrifuging and discarding the supernatant each time. After the third wash, the mixture was

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