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Inhibition or ablation of transglutaminase 2 impairs astrocyte migration

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ABSTRACT

Astrocytes play numerous complex roles that support and facilitate the function of neurons. Further, when there is an injury to the central nervous system (CNS) they can both facilitate or ameliorate functional recovery depending on the location and severity of the injury. When a CNS injury is relatively severe a glial scar is formed, which is primarily composed of astrocytes. The glial scar can be both beneficial, by limiting inflammation, and detrimental, by preventing neuronal projections, to functional recovery. Thus, understanding the processes and proteins that regulate astrocyte migration in response to injury is still of fundamental importance. One protein that is likely involved in astrocyte migration is transglutaminase 2 (TG2); a multifunctional protein expressed ubiquitously throughout the brain. Its functions include transamidation and GTPase activity, among others, and previous studies have implicated TG2 as a regulator of migration. Therefore, we examined the role of TG2 in primary astrocyte migration subsequent to injury. Using wild type or $TG2^{-/-}$ astrocytes, we manipulated the different functions and conformation of TG2 with novel irreversible inhibitors or mutant versions of the protein. Results showed that both inhibition and ablation of TG2 in primary astrocytes significantly inhibit migration. Additionally, we show that the deficiency in migration caused by deletion of TG2 can only be rescued with the native protein and not with mutants. Finally, the addition of TGFβ rescued the migration deficiency independent of TG2. Taken together, our study shows that transamidation and GTP/GDP-binding are necessary for inhibiting astrocyte migration and it is TGFβ independent.

1. Introduction

Astrocytes are the most abundant cell type in the central nervous system (CNS). In addition to providing essential metabolic and structural support to neurons, astrocytes are active in many other homeostatic processes in the CNS [1]. During pathological events astrocytes can respond in a number of different ways, which can be both detrimental and beneficial to functional recovery. For example, in mild to moderate CNS injuries, reactive astrocytes can protect the tissue and preserve function. However, when the injuries are more severe, the resulting glial scar that plays a role in sequestering inflammatory cells also prevents neuronal projections from traversing the injured area, thus limiting functional recovery [2–4]. Often at the glial scar there is an increase in the number of astrocytes surrounding the injury site. This increase can partly be due to migrating astrocytes [2,4]. Because of the importance of reactive astrocyte migration in response to injury, understanding the molecular mechanisms that regulate these processes is of fundamental importance.

Transglutaminase 2 (TG2) is a multifunctional protein that is expressed in numerous cell types (including astrocytes) and has been implicated as a regulator of migration in several clonal cell types including HEK, NIH 3T3 and cancer cell lines [5,6]. TG2 can catalyze calcium-dependent transamidating reactions, bind and hydrolyze GTP, and act as a scaffold protein, among other functions [5]. TG2 undergoes significant conformational changes which are reciprocally regulated by calcium and GTP binding. In high calcium

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environments, TG2 is transamidation active as it exists in a more open conformation, while GTP binding causes TG2 to take on a more closed conformation, which prevents it from catalyzing transamidation reactions [5]. These conformational changes appear to be crucial for mediating the localization, interactions, and functioning of TG2.

It has been reported that TG2 can both facilitate and inhibit cell migration [5,7,8]. Overexpression of TG2 in HEK cells, as well as in a cancer cell line, resulted in a significant inhibition of migration [9]. In contrast, overexpression of TG2 in a human neuroblastoma cell line resulted in increased migration [10]. The reasons for these different effects of TG2 on migration are likely due in part to the fact that TG2 plays different roles in a context and cell-type specific manner and is thus likely to be modulating different targets in the various models [5,6]. For example, TG2 has been shown to modulate integrin and MAP kinase pathways in certain cell types, which can control actin dynamics to facilitate cellular migration [11,12]. Other studies have shown that extracellular TG2 may modulate cell migration by acting as an intermediate between the extracellular matrix (ECM; fibronectin) and cell contacts (integrin) [13]. Moreover, intracellular TG2 has been shown to interact with JNK and p38, both of which can be activated though the TGFβ receptors and enhance migration [12,14]. In astrocytes, activation of the TGFβ receptors increases astrocyte mobility partly due to activation of the integrin and MAP kinase pathway, which modulates their morphology and movement [15].

Given the importance of astrocyte migration subsequent to CNS injuries, the focus of this study was on determining the role of TG2 in this process. Previously, TG2's role in migration of astrocytes has only been explored in the context of multiple sclerosis and not in response to injury [16]. In this study we examine how deletion of TG2 from astrocytes affected injury-induced migration, and further how modulation of TG2's activity/conformation impacted astrocyte migration using novel TG2 irreversible inhibitors.

2. Methods and materials

2.1. Animals/primary cell culture

Animals were housed and euthanized in accordance with guidelines established by the University of Rochester Committee on Animal Resources. The studies were carried out with approval from the Institutional Animal Care and Use Committee.

Wild type (WT) and TG2$^{-/-}$ mice [17] on a C57Bl/6 background were used to prepare primary astrocytes as described previously [18]. In brief, cortices were harvested at post-natal day 0 from WT C57BL/6 or TG2$^{-/-}$ mouse pups. Hemispheres were then mechanically dissociated and plated onto culture dishes in MEM media supplemented with 10% FBS (Atlanta Biologicals), 6 g/L glucose, 1 mM sodium pyruvate and 100 μg/mL primocin (Fisher). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The following day, the plates were shaken to remove non-adherent cells and the remaining adherent cells were then rinsed once with MEM media. Cells were then maintained in 10% FBS MEM for 5–7 days until they reached confluency, upon which cells were frozen in media containing 10% FBS/10% DMSO and stored in liquid nitrogen for future use. This culturing technique yields >95% astrocytes [18]. For experiments, astrocytes were thawed, re-plated and maintained in 10% FBS MEM.

2.2. Scratch assay

WT or TG2$^{-/-}$ astrocytes were plated on 12- or 24-well plates and maintained under normal culture conditions until they reached confluency. A p10 sterile pipette tip was used to make one vertical scratch in each well. Three pictures were taken at 0, 24, and 48 h after the scratch, using a 100× magnification with the Zeiss LED microscope in bright field, for each well. ImageJ software was used to calculate the distance for each picture. The average of three pictures for each well was used as the final result for each independent experiment.

2.3. Astrocyte viability

WT astrocytes plated on 12-well plates were treated with 5 or 10 μM of VA4 and VA5 or DMSO control for 24 and 48 h. VA4 and VA5 are selective TG2 inhibitors that stabilize TG2 in an open conformation [19,20]. Cell viability was assessed with the resazurin assay as previously described [18]. In brief, a final concentration of 0.05 mg/mL of resazurin was added to each well. Plates were then incubated at 37 °C, 5% CO_2 for 30 min and reduction of resazurin to resorufin was assessed using a fluorescence microplate reader (Biotek) with 540-nm excitation and 590-nm emission filters. Data were normalized to DMSO control.

2.4. Cytochemistry

WT and TG2$^{-/-}$ astrocytes were plated on 24-well plates and allowed to reach confluency prior to scratching as described above. After 0, 24, and 48 h post-scratch, cells were gently rinsed with PBS prior to fixation with 3.7% paraformaldehyde for 15 min at room temperature. Cells were then washed and blocked with 3% BSA (Fisher) in ddH_2O. Cells were incubated with 1:20 Phalloidin Alexa 647 (Fisher) and 1:2000 Hoechst (Fisher) for 20 min in PBS and subsequently rinsed twice with PBS. Imaging was done with a Zeiss LED microscope using 430 and 590 nm filters. Pictures were taken with 100× magnification. Fluorescence intensity of images was analyzed with Adobe Photoshop. Quantification of plasma membrane directional actin orientation was carried out by a person blinded to the genotype or treatment group. Quantification was done counting the cells with increased fluorescence at the leading edge of migrating cells in a 100× magnification pictures.

2.5. Immunoblotting

Two million TG2$^{-/-}$ astrocytes were nucleofected as directed by the manufacturer (Lonza) with 4 μg of pcDNA, 2 μg human TG2, 4 μg W241A, or 5 μg R580A. Nucleofected TG2$^{-/-}$ or WT astrocytes were then plated onto 60-mm dishes and harvested as previously described [19,21]. In brief, cells were collected after 4 days of nucleofection, protein concentrations determined and 40 μg of protein was run on a 12% SDS-polyacrylamide gel. After transferring, nitrocellulose membranes were probed with 1:5000 of a rabbit monoclonal α-tubulin from Cell Signaling and 1:2500 of the rat monoclonal TG2 antibody TGMO1 [22] followed by incubation with the appropriate secondary antibodies and development of the blots with chemiluminescence. Immunoblots were quantitated using ImageJ software.

2.6. Treatment paradigms

Three different treatment paradigms were used for the scratch assay. First, WT astrocytes were treated with 5 ng/mL of TGFβ1 (R&D Systems), 5 μM VA4 or VA5, or DMSO control in serum free MEM media, supplemented with 6 g/L glucose, 1 mM sodium pyruvate and 100 μg/mL primocin, for 24 and 48 h. Second, 24 h after nucleofection (as described above), TG2$^{-/-}$ astrocytes were transferred to serum free MEM media, scratched, and imaged at 0, 24 and 48 h. Finally, TG2$^{-/-}$ astrocytes were treated with 5 ng/mL of TGFβ1, 10 ng/mL TNFα (Fitzgerald Industries International), or 5 ng/

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