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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.

Keywords

transglutaminase 2; astrocytes; migration; TGFβ; transamidation

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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 [2,3,4]. 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 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 through the TGF β receptors and enhance migration [12,14]. In astrocytes, activation of the TGF β receptors increases astrocyte mobility partly due to activation of 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.

Methods and Materials

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% CO₂. 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.

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 hr after the scratch, using a 100X 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.

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 hr. 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% CO₂ 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.

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 hr 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 ddH₂O. 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 100X 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 100X magnification pictures.

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 and WT astrocytes. Cells 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.

Treatment Paradigms

Three different treatment paradigms were used for the scratch assay. First, WT astrocytes were treated with 5 ng/mL of TGFβ, 5 µM VA4 or VA5, or DMSO control in serum free MEM media, supplemented with 6 g/L glucose, 1mM sodium pyruvate and 100 µg/mL primocin, for 24 and 48 hr. Second, after 24 hr of nucleofection (as described above), TG2^{-/-} astrocytes were transferred to serum free MEM media, scratched, and imaged at 0, 24 and 48 hr. Finally, TG2^{-/-} astrocytes were treated with 5 ng/mL of TGFβ1 (R&D Systems), 10 ng/mL TNFα (Fitzgerald Industries International), or 5 ng/mL IL1β (Shenandoah Biotechnology) in serum free MEM conditions.

Results

Inhibition or ablation of TG2 inhibits astrocyte migration

In order to begin to understand the role of TG2 in mediating astrocyte survival and migration, we used two irreversible TG2 inhibitors, VA4 and VA5 which have recently been described [19]. We first tested the viability of primary WT mouse astrocytes when treated with VA4 and VA5. Results showed no toxicity of the inhibitors when treated with 5 or 10 µM VA4 or VA5 (Figure 1A). Interestingly, treatment with VA5 resulted in an increase in astrocyte viability at both 24 and 48 hr as measured by the reduction of resazurin to resorufin (Figure 1A). Using the working concentration of 5 µM, treatment with VA5 resulted in a significant inhibition of migration when used in a primary WT astrocyte migration assay (Figure 1B and C). Treatment with either VA4 or VA5 resulted in a significant decrease in migration when compared to the TGFβ positive control (Figure 1B and 1C). To further understand the role of TG2 in astrocytic migration, WT and TG2^{-/-} astrocytes were subjected to a scratch wound assay and the ability to migrate was compared. Results showed a significant decrease in migration in TG2^{-/-} astrocytes when compared to WT astrocytes (Figure 2A). This migration deficiency of the TG2^{-/-} astrocytes was rescued

only when TG2 was re-introduced (Figure 2B, C and D); neither a mutant that does not bind GTP (R580A), nor a transamidating-inactive mutant (W241A), were able to rescue the phenotype (Figure 2C and D) [21].

TGF β activates astrocyte migration independently of TG2

Inflammatory cytokines, such as TGF β , have been previously shown to increase migration in WT astrocytes [15,23]. Additionally, there is evidence that inflammatory cytokines IL1 β and TNF α inhibit migration in WT astrocytes [24]. To determine if the TG2^{-/-} astrocyte deficiency in migration could be rescued with cytokines or further inhibited, we treated TG2^{-/-} astrocytes with 5 ng/mL TGF β 1, 10 ng/mL TNF α , or 5 ng/mL IL1 β and measured migration. Results showed a significant change (increase) in wound closure only in response to 5 ng/mL of TGF β 1 (Figure 3A and B). TGF β 1 is a known activator of TGF β receptors, which signal through the MAP kinase pathway to modulate actin dynamics [25]. Since the migration of TG2^{-/-} could be rescued with TGF β treatment, the actin machinery is not impaired. Thus, we wanted to understand if TGF β treatment facilitated actin re-organization in TG2^{-/-} astrocytes. Results showed no difference in total mean fluorescence of F-actin filaments in WT or TG2^{-/-} serum free (SF) and 5 ng/mL TGF β 1 treated cells (Figure 3B and C). Furthermore, there was no difference between the treatment groups in phalloidin-positive cells at the leading edge between the treatment groups (Figure 3D).

Discussion

Astrocyte migration plays a critical role in CNS injury as it contributes to the formation of the glial scar [2,4]. Although there is currently not a consensus as to the relative contribution of migratory astrocytes to scar formation, understanding the processes that regulate astrocyte migration in response to injury is still of fundamental importance as it could help direct the development of potential therapeutic interventions. Indeed, the differences in the extent to which migrating astrocytes contribute to scarring is likely dependent, in part, on the type and location of the injury and thus, in some instances, attenuating the migratory phenotype could have significant beneficial effects. In our study, we focused on the role of TG2 in modulating astrocyte migration in response to injury. Using two novel irreversible TG2 inhibitors, VA4 and VA5, we were able to inhibit transamidation activity and GTP binding, and stabilize TG2's conformation in a more open state [19]. Interestingly, in our studies, only VA5 was able to significantly inhibit astrocyte migration (Figure 1). This could be, in part, due to subtle differences in conformation TG2 partakes when bound to VA5 compared to VA4. TG2's conformation has been shown to play a significant role in altering proliferation and migration of cells due to changes in signaling partners [5,6]. Some groups have shown that extracellular TG2's transamidating activity is not required for integrin-induced migration but conformation is important [11]. It is known that TG2 is localized both intracellular and extracellular in primary astrocytes [16], and the van Dam group have shown that inhibition of TG2 with KC009 can decrease migration of primary astrocytes partly due to a decrease in extracellular matrix crosslinking [16]. Thus further examination of TG2's activities during migration was needed in order to assess if conformation was the key.

After establishing that TG2^{-/-} astrocytes have an impairment in their ability to migrate when compared to WT control (Figure 2), we assessed if adding back native TG2, mutant R580A (transamidation active only), or mutant W241A (GTPase active only) to TG2^{-/-} astrocytes, could rescue the phenotype. Our results showed that only native TG2 is able to rescue the migration deficiency phenotype. Along with the van Dam group, we were able to confirm that astrocytic migration is TG2-activity dependent. Furthermore, our studies suggest that both transamidation and GTPase activities are needed for migration (Figure 2), which has also been observed in TG2-mediated fibroblast migration [8]. Taken together, these results suggest that TG2 may be not be modulating the integrin pathway [11]. In order to further understand which signaling pathway TG2 may be working through, we activated different receptors that have been shown to be important in CNS injury (IL1 β , TGF β , TNF α), astrocytic migration (TGF β , TNF α), and/or known TG2 signaling pathways [23,24,26,27]. Both IL1 β and TGF β are well known activators of the MAP kinase pathway [25,28]. And, IL1 β and TNF α are known activators of the NF- κ B pathway [29]. Additionally, TGF β activates astrocytic migration, while TNF α inhibits it [23,24]. Since TGF β increased migration independent of TG2, and both IL1 β and TNF α are inhibitory cytokines of migration, TG2 may not be acting through the MAP kinase pathway (Figure 3).

We wanted to understand why TGF β was able to increase migration in TG2^{-/-} astrocytes. Since the activation of TGF β through the non-canonical pathway can activate MAP kinases to modulate actin dynamics, we decided to assess if the actin machinery of TG2^{-/-} astrocytes was impaired in serum free conditions compared to TGF β conditions. The actin fluorescence intensity was the same between the groups, and astrocytes had the same ability to re-direct F-actin to the leading edge of the cells when compared to control (Figure 3). This confirms that the TGF β -induced increase in migration of TG2^{-/-} astrocytes is not due to modulation of actin dynamics.

In this study we have shown for the first time the importance of TG2 in astrocyte migration following an injury. Specifically, TG2 appears to require both its transamidation and GTPase function in order to facilitate astrocyte migration. This deficiency in migration is also seen when WT astrocytes were inhibited with VA5, a novel irreversible TG2 inhibitor, that inhibits both transamidation and GTP binding by altering the protein's conformation [19]. Finally, we have shown that this deficiency can be bypassed by activation of TGF β receptors, suggesting that TG2 does not facilitate migration through the MAP kinase pathway. Further research is needed in order to characterize the signaling pathway(s) by which TG2 facilitates astrocyte migration.

Acknowledgments

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Highlights

- Ablation of transglutaminase 2 impairs astrocyte migration subsequent to injury
- Astrocyte migration can only be restored with catalytically active transglutaminase 2
- Inhibition of transglutaminase 2 transamidating activity and GTP binding impairs injury-induced migration.
- The facilitation of astrocyte migration of transglutaminase 2 is likely independent of the TGF β signaling pathway

Figure 1. Inhibition of TG2 decreases migration of primary WT astrocytes

A. Cell viability was measured after WT astrocytes were incubated for 24 or 48 hr with TG2 inhibitors VA4 and VA5, at the concentrations indicated. $n=3$ * $p<0.05$; ** $p<0.002$. **B.** Representative images of the scratch assay used to assess effectiveness of TG2 inhibitors, VA4 and VA5, in decreasing WT astrocyte migration. Pictures were taken at 200X magnification in bright field. **C.** Quantification of B. Data were normalized to each individual 0 hr control. $n=5$ independent experiments * $p<0.05$.

Figure 2. TG2^{-/-} astrocyte decrease in migration can only be rescued with wild type TG2
A. Scratch assay used to assess differences in migration between WT and TG2^{-/-} astrocytes. Data was normalized to each individual 0 hr control. n=3 separate experiments, *p<0.05. **B.** Immunoblot of nucleofected TG2^{-/-} astrocytes with pcDNA, TG2, R580A mutant, and W241A mutant. **C.** Scratch assay used to determine if adding back TG2 or mutant TG2 could rescue the migration deficiency. Pictures were taken at 100X magnification in bright field. Data was normalized to each individual 0 hr control. n=3 separate experiments, *p<0.05.

Figure 3. TGF β rescues TG2^{-/-} astrocyte migration deficiency

A. Quantification of a scratch assay to assess if TGF β , IL1 β , or TNF α could rescue the TG2^{-/-} migration deficiency. Data was normalized to each individual 0 hr control. n=5 *p<0.05. **B.** Representative images from the scratch assay of WT and TG2^{-/-} astrocytes incubated in the absence or presence of 5 ng/mL TGF β 1. Cells were stained with phalloidin to evaluate the actin cytoskeleton and counterstained with Hoechst for nucleus staining. Pictures were taken at 100X magnification. **C.** Quantification of fluorescence mean of B. n=4.