

***Astrocyte-derived tissue Transglutaminase
interacts with fibronectin:
a role in glia adhesion and migration?***

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Abstract

An important neuropathological feature of brain injury and neuroinflammation, including Multiple Sclerosis (MS), is the formation of an astroglial scar. Astroglial scar formation is facilitated by the interaction between astrocytes and extracellular matrix proteins (ECM) such as fibronectin. Since there is evidence indicating that glial scars strongly inhibit both axon growth and (re)myelination in brain lesions, it is important to understand the factors that mediate scar formation.

Tissue Transglutaminase (TG2) is a multifunctional enzyme with a ubiquitous tissue distribution, being clearly present within the brain. It has been shown that inflammatory cytokines can induce TG2 activity. In addition, TG2 can mediate cell adhesion and migration and it binds fibronectin with high affinity. We therefore hypothesized that TG2 is involved in astrocyte-ECM interactions.

Our studies using primary rat astrocytes show that intracellular and cell surface expression and activity of TG2 is increased after treatment with pro-inflammatory cytokines. TG2 on the surface of astrocytes interacts with fibronectin and is involved in astrocytic adhesion to fibronectin. TG2 is an essential factor in stimulating focal adhesion formation which is necessary for interaction of astrocytes with the ECM. We conclude that astrocyte-derived surface TG2 contributes to the interaction between astrocytes and fibronectin, thereby regulating ECM remodeling and possibly glial scarring.

Introduction

Astrocytes within the brain comprise the largest glial cell population. These cells are considered to be important for maintaining an environment in which neurons, other glial cell types and the brain endothelium function and interact properly.²⁷² Injury to the central nervous system (CNS) often results in a characteristic astroglial response, i.e. the astrocytes become activated, migrate, and form a dense network of hypertrophic cells, the astroglial scar.^{312, 350, 397} Additional cell types including macrophages, microglia, oligodendrocytes, and meningeal fibroblasts contribute to the glial scar,¹⁰³ but astrocytes predominate and are the focus of the present study. The astroglial scar consists of a fine meshwork of astrocyte processes strongly interwoven and bound together by tight and gap junctions, surrounded by extracellular matrix (ECM).^{36, 103, 309}

In situations of chronic neuroinflammation, e.g. Multiple Sclerosis (MS), when inflammatory cytokines are produced and released within the CNS,²⁹ sustained and excessive deposition of ECM proteins such as fibronectin and activation of astroglial cells can create an environment in which an astroglial scar is formed. Moreover, the cytokine interleukin-1 β (IL-1 β) has been shown to promote the reactive astrocytic phenotype and adhesion of astrocytes onto fibronectin or laminin.¹⁷⁴ The astroglial scar acts as a physical or biochemical barrier that impedes tissue repair.¹⁰³ For instance, a reduction in oligodendrocyte precursor cell (OPC) migration and differentiation has been described,^{79, 342, 343} as well as attenuated myelination of axons by oligodendrocytes.¹⁰³

Thus far, studies on astrogliosis and down-stream mechanisms involved in the interaction between astrocytes and ECM molecules focus on relatively acute (hours) effects. However, patients suffering from brain injury and/or neuroinflammation experience long-term consequences of their disease, i.e. impaired regeneration. In that respect, we are interested in the role of tissue Transglutaminase (tTG or TG2) in mediating cytokine-induced activation of astrocytes and interaction with fibronectin after (relatively) long-term cytokine treatment. It has been shown that upon treatment of different cell types with cytokines, TG2 expression and activity was elevated for a longer period of time, i.e. up to 7 days^{188, 304} which may be more relevant for the human situation.

TG2 is the ubiquitous member of a family of transglutaminase enzymes. Its functional role remains to be fully established, but TG2 is well known for its ability to posttranslationally modify proteins in a calcium-dependent manner. TG2 can cross-link proteins, amidate or deamidate proteins, it can bind and hydrolyse GTP to mediate cell signaling, and it has isopeptidase activity.¹⁰⁶ TG2 is mainly expressed in the cytoplasm, but it can also be present in the extracellular matrix.¹⁰⁶ In addition, it has been shown that TG2 is present on the surface of monocytes,⁹ monocyte-derived dendritic cells and macrophages,¹⁵⁴ endothelial cells¹²⁸ and fibroblasts.³⁶⁶ Since TG2 is present on the cell surface of these cell-types and has a fibronectin binding site located in the N-terminal domain, a prominent role for TG2 in stimulating cell adhesion has been put forward.^{9, 366} Besides the interaction of TG2 with fibronectin to stimulate cell adhesion, it is involved in numerous other adhesion-dependent phenomena including cell migration, extracellular matrix assembly, and signaling.³⁹² Cytoskeletal reorganization and focal adhesion dissolution^{346, 347} are required to mediate those processes.

Based on these considerations, in our quest to unravel the mechanism(s) that contribute

to relatively long-term astroglia activation and astroglial scar formation, we hypothesize that the enzyme TG2 is an important player. To investigate this issue, we used primary cultured rat astrocytes, and observed cytokine-induced enhanced intracellular and surface expression of TG2. Furthermore, TG2-mediated cross-linking activity resulted in enhanced astrocyte adhesion to and migrate across fibronectin, most likely mediated via effects on focal adhesion-related vinculin expression.

Materials and Methods

Primary rat astrocyte culture and cytokine treatment

Primary cultures of astrocytes were prepared from newborn (2 day old) Dark Agouti rats (Harlan CPB, Zeist, The Netherlands) as described.²⁴² In short, cerebral cortices were cleared from adhering meninges and blood vessels and dissociated using 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS). Cells were plated in poly-L-lysine (15 mg/ml; Sigma-Aldrich) coated T75 culture flasks (Nunc, Hamstrop, Denmark) and incubated at 37°C in humidified air containing 5% CO₂. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)-F10 (Gibco, Life Technologies, Breda, The Netherlands), supplemented with 10% v/v heat-inactivated fetal calf serum (FCS) (Gibco), 2 mM L-glutamine (Sigma-Aldrich), 37.5 U/ml streptomycin (Sigma-Aldrich), and 100 U/ml penicillin (Sigma-Aldrich). The medium was changed 1 day after seeding. After 8 days of culture, pure astrocytes were obtained by shaking the flasks at 37°C on a rotary platform (Heidolph Unimax 2010) at 240 rpm for 16 h to remove microglia and oligodendrocyte progenitors. Fresh medium was added to the flasks and cells were ready for use in further experiments. Primary astrocytes were used for a maximum of 5 passages.

For experiments with primary rat astrocytes, cells were plated onto wells that were coated with 2 µg/mm² fibronectin (Fn, Sigma-Aldrich) for 1 h at 37°C. Astrocytes were cultured in medium alone (control) or the presence of rat recombinant (rr) TNFα (Pharmingen, 50 ng/ml), rr IFNγ (R&D systems, 50 ng/ml), rr IL-1β, (Glaxo, 50 ng/ml) or combinations of the various cytokines (50 ng/ml each) for 48 h.

To determine the role of TG2 in adhesion and migration onto Fn, astrocytes were co-incubated with 0.5 mM KCC009, a specific irreversible inhibitor of TG2 activity,⁶⁸ in 0.2% DMSO or 0.2% DMSO only (vehicle).

TG2 immunocytochemistry

Primary astrocytes were cultured in Fn coated 8-well chamber slides (Nunc) at 37°C. After 24 h, cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.6) for 15 min and subsequently rinsed with PBS. The cells were incubated with mouse anti-TG2 (Ab3, Labvision, 10 µg/ml) in PBS/2% bovine serum albumin (Sigma-Aldrich) for 4 h at 4°C. After washes in PBS, the cells were incubated with biotinylated donkey anti mouse IgG's (Jackson laboratories, 1:500) for 1 h at room temperature (RT) followed by 1 h incubation with Alexa Fluor-488-coupled streptavidin (Invitrogen, 1:400) at RT. Cells were washed with PBS and slides were embedded in vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Pictures were taken using a Colorview II digital camera (Olympus, Soft Imaging System, Munster, Germany) and Cell*F software (Olympus Soft Imaging Solutions GmbH).

Quantitative RT-PCR

For quantitative RT-PCR, 1×10^6 astrocytes were homogenized in Trizol reagent (Invitrogen, Carlsbad, USA) and total RNA was isolated as described by the manufacturer. RNA concentration and purity was determined by measuring the absorbance at 260 nm and 280 nm in a microtiter plate reader (Spectramax 250, Molecular Devices). One μ l of RNA was reverse transcribed into cDNA using the Reverse Transcription System (Promega, Madison, WI, USA) with oligo-dT primers and AMV enzyme, according to the manufacturer's instructions. The PCR reaction was carried out at 42°C for 30 min, followed by deactivation of the enzyme at 95°C for 5 min and 4°C for 5 min. For the PCR reaction, the SYBR Green PCR Core reagents kit (Applied Biosystems, Foster City, CA, USA) was used. Intron-spanning primers were designed using Primer Express Software (Applied Biosystems) and purchased from Eurogentec (Seraing, Belgium). Amplification of cDNA was performed in MicroAmp Optical 96-well Reaction Plates (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reaction mixture (20 μ l) was composed of 1x SYBR Green buffer, 3 mM $MgCl_2$, 875 μ M dNTP mix, 0.3 U AmpliTaq gold, 0.12 U Amperase UNG, 15 pmol of each primer (GAPDH forward: 5' TCAAGGGCATCCTGGGCTAC 3', reverse: 5' CGTCAAGGTGGAGGAGTGG 3' and TG2 forward: 5' GGCCTGACCAAGGAACAGAAG 3', reverse: 5' CAATATCAGTCGGGAACAGGTC 3'), 12.5 ng cDNA and nuclease free H_2O . The reaction conditions were an initial 2 min at 50°C, followed by 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 59°C. The mRNA expression levels were quantified relatively to the level of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) using the following calculation: $2^{-(Threshold\ cycle\ of\ target\ mRNA - Threshold\ cycle\ of\ GAPDH)} \times 100\%$.

Western blotting

Astrocytes were homogenized in ice-cold lysis buffer containing 10 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μ M PMSF, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin and 10 μ g/ml aprotinin (all from Sigma-Aldrich). Homogenates were cleared by centrifugation (14,000 rpm for 30 min at 4°C) and protein concentrations of supernatants were determined by the BCA method (Pierce Biotechnology, Perbio Science, Etten-Leur, NL). Of each sample, 10 μ g of protein was subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). Membranes were incubated with primary mouse anti-TG2 (Ab3, Labvision, 1:1,000), mouse anti-vinculin (Abcam, Cambridge, UK, 1:400) or mouse anti- β -actin (Abcam, 1:2,000). For subsequent antigen detection, blots were incubated for 2 h with corresponding goat anti-mouse or donkey anti-rabbit Immunoglobulins/HRP (Dako, Glostrup, Denmark, 1:10,000). Bands were visualized using the enhanced chemiluminescence (ECL) detection system SuperSignal West Dura (Pierce Biotechnology) and a Chemidoc image capture system (Bio-Rad, Veenendaal, The Netherlands). Signal intensity of the bands was semi-quantified using Quantity One software (Bio-Rad, Veenendaal, The Netherlands).

TG2 ELISA

TG2 protein levels were measured in cell lysates using a sandwich enzyme-linked immunosorbent assay (ELISA) specific for TG2 as described previously.⁴⁷ Briefly, astrocytes were homogenized in ice-cold lysis buffer as described for western blot. Homogenates

were cleared by centrifugation (14,000 rpm for 30 min at 4°C) and protein concentrations of supernatants were determined by the BCA method (Pierce Biotechnology, Perbio Science, Etten-Leur, NL). Of each sample, 10 µg of protein was loaded into the assay. An immunoaffinity-purified polyclonal goat anti-TG2 antibody (Upstate, Millipore, USA) was used as coating antibody and a monoclonal mouse anti-TG2 antibody (Ab2, Labvision, Fremont, CA, USA) was used as detecting antibody. Recombinant human TG2 (Zedira Biotec GmbH, Darmstadt, Germany) was used as standard.

TG activity assay

To measure TG activity, astrocytes were treated with 0.5 mM KCC009 or vehicle at 37°C for 1 h. Subsequently, cells were homogenized in ice-cold lysis buffer as described for western blot. Homogenates were centrifuged for 30 min at 14,000 rpm at 4°C, and protein concentrations of supernatants were determined by the BCA method (Pierce Biotechnology). Activity was measured by using the TG Covtest TCMA (Transglutaminase Colorimetric Microassay; Covalab, Villeurbanne, France) following manufacturer's protocol.³²³ In short, immobilized CBZ-Gln-Gly was coated onto the wells as the first TG substrate. Subsequently, 10 µg of protein from each sample was added/well, followed by addition of biotinylated cadaverine as a second substrate. After 30 min incubation at 37°C, plates were washed with Tween-20 buffered saline (TTBS) and streptavidin-labeled peroxidase (HRP) diluted in TTBS was added to the wells for 15 min. After washing, peroxidase activity was revealed using 100 µl of 0.01% H₂O₂ as HRP substrate and (0.1 mg/ml) tetramethyl benzidine as electron acceptor (chromogen). The reaction was stopped by the addition of 50 µl of 2.5 N H₂SO₄. TG activity was detected by absorbance measurement of streptavidin-labeled peroxidase activity in each well on a microplate reader (SpectraMax 250, Molecular Devices) at 450 nm. Purified guinea pig TG2 (Zedira Biotec GmbH) was used as standard.

Immunofluorescent analysis of TG2 on the cell surface

For immunofluorescent surface labeling of TG2, astrocytes were washed with TBS and incubated on ice with a mouse monoclonal anti-TG2 antibody (Ab1, Labvision, 1:1,000) for 2 h. Cells were washed with TBS, followed by incubation with Alexa Fluor-488 (Invitrogen, 1:400) to detect TG2 immunoreactivity. Subsequently, cells were washed with TBS and fixed with 4% paraformaldehyde (PFA). After washing with H₂O, sections were embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and examined on a Leica confocal laser scanning microscope (Leica, Rijswijk, The Netherlands).

Cell surface biotinylation and immunoprecipitation

To determine TG2 expression on the cell surface, cells were plated in Fn coated wells of a 6-well plate, (2.5x10⁵ cells/well, Nunc) and allowed to adhere overnight (o/n). Cells were incubated in the presence of cytokines (50 ng/ml) for 48 h. Subsequently, cells were washed with ice-cold PBS. Cells were surface biotinylated by incubating for 20 min with 0.2 mg/ml Sulfo-NH-Biotin (Pierce) in PBS at 4°C. The reaction was stopped by addition of 20 mM Tris-HCl, pH 7.5. Surface-biotinylated cells were washed with in PBS at 4°C and lysed in ice-cold lysis buffer as described for western blot. Cell lysates were cleared by centrifugation (14,000 rpm for 30 min at 4°C). Then, 1 mg of total cell protein was taken

for each immunoprecipitation with an anti-TG2 antibody (Ab3, Labvision, 2 μg antibody/sample). Levels of surface TG2 were detected using gelelectrophoresis and western blot as described above.

Flow cytometry

To study TG2 activity on the surface of astrocytes after treatment with various cytokines and the effect of inhibition of TG2 activity, biotin-cadaverine incorporation on the cell surface was measured using flow cytometry (FACS). Cadaverine is a substrate for TG2,¹⁹⁸ and measurement of biotinylated cadaverine by FACS analysis reflects TG2 activity on the cell surface. Therefore, astrocytes were plated onto Fn coated wells and cultured in the presence of TNF α , IL-1 β or the combination of both for 48 h. After incubation, cells were washed with PBS containing 0.1% glucose and detached with 10 mM EDTA in PBS/0.1% glucose. Subsequently, cells were counted and 1.5×10^5 cells were resuspended in 1 ml AC-buffer containing PBS/0.1% glucose containing 1.2 mM CaCl₂ and 0.5 mM MgCl₂. Then, cells were pre-incubated with KCC009 or vehicle in AC-buffer at 37°C for 15 min. Subsequently, biotin-cadaverine (Pierce EZ Link pentylamine-biotin, BAP) was added to the cells in a final concentration of 0.2 mM and cells were incubated for 1 h at 37°C in the presence of vehicle or KCC009. After incubation, cells were placed on ice, washed with AC-buffer containing 0.1% BSA and subsequently stained with streptavidin-Alexa Fluor-488 (Invitrogen, 1:300) at 4°C for 30 min. Cells were washed with AC-buffer containing 0.1% BSA and resuspended in AC-buffer/0.1% BSA containing 1 $\mu\text{g}/\text{ml}$ propidium iodide (PI) to stain apoptotic cells. Then, flow cytometry was performed on a FACScan (BD Biosciences) and analyzed using WinMDI software (BD Biosciences). Analysis gates were set on propidium iodide (PI) negative cells. Three independent experiments were performed and in each experiment, BAP+vehicle treated control cells were set at 100%.

BAP incorporation assay

To study the role of TG2 in astrocyte-Fn interaction, a biotin-cadaverine incorporation assay was performed in which the incorporation of biotin-cadaverine into Fn was measured.¹⁸⁰ In short, 48 h-cytokine treated rat astrocytes were detached with 10 mM EDTA, resuspended in fresh medium and plated onto Fn coated 96-well plates (50 $\mu\text{l}/\text{well}$ corresponding to $\sim 3.5 \times 10^4$ cells/well) in the presence of 0.5 mM KCC009 in 0.2% DMSO or 0.2% DMSO only (vehicle). Biotin-cadaverine was added directly (50 μl 0.1 mM, Pierce EZ Link pentylamine-biotin) and cells were incubated for 1 h at 37°C. Subsequently, cells were washed with PBS containing 3 mM EDTA and then incubated for 20 min with 0.1% sodiumdeoxycholate (Sigma) in PBS containing 3 mM EDTA. Supernatant and cells were removed and the plate was washed with 0.1 M Tris-HCl (pH 7.4). Then, the plate was incubated for 1 h at 37°C with streptavidin poly-HRP (1:10,000 in Tris/HCl). After washing with Tris/HCl, peroxidase activity was revealed using 0.01% H₂O₂ and 0.1 mg/ml tetramethyl benzidine (chromogen). The reaction was stopped by the addition of 50 μl of 2.5 N H₂SO₄ and OD was measured on a microplate reader (SpectraMax 250, Molecular Devices) at 450 nm. Three independent experiments with duplicate measurements were performed for each treatment.

Astrocyte adhesion assay

Astrocyte adhesion was studied as described.²² In short, 96-well plates were coated with Fn (Sigma-Aldrich) for 1 h at 37°C. Primary rat astrocytes that had been treated with cytokines for 48 h were detached with 2 mM EDTA and plated onto Fn-coated wells (5×10^4 cells/well) in serum free medium. Cells were allowed to adhere for 3 h at 37°C in the presence of 0.5 mM KCC009 or vehicle. After 3 h, cells were washed with PBS and fixed with 4% PFA in 0.1 M phosphate buffer for 30 min at RT. Cells were stained with 100 μ l crystal violet solution (0.5 gr/100 ml 70% EtOH) for 40 min. Cells were washed with PBS and crystal violet was extracted from the cells with 100 μ l 30% acetic acid. The absorbance was measured on a microplate reader (SpectraMax 250, Molecular Devices) at 540 nm. Three independent experiments with duplicate measurements were performed for each treatment.

Astrocyte viability measurements

The effect of KCC009 treatment on cell viability was determined by PI exclusion assay. Rat astrocytes were plated in a 96-well plate (20,000 cells/well) and allowed to adhere for 24 h in serum free medium. Medium was replaced with PBS containing 0.5 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1% glucose, 40 μ g/ml PI (Sigma) and 0.5 mM KCC009 or vehicle. During an incubation period of 24 h at 37°C, PI fluorescence was measured at 30 min intervals, using a Fluostar OPTIMA microplate reader with an excitation wavelength of 544 nm and an emission of 612 nm. Average slope/minute was measured per 30 min interval and average slope/minute in 24 h was determined (F-average). A total of 160 μ M digitonin was then added for 20 min to permeabilize all cells and fluorescence measurements were performed to obtain a maximal fluorescent signal (Fmax). Percentage viability was calculated as $100 - (F\text{-average}/F\text{max-blank}) \times 100\%$.³²⁵

Astrocyte migration assay

Astrocyte migration was studied as described.¹⁷⁴ In short, permanox Chamberslides (4-wells, Nunc) were coated with 2 μ g/cm² Fn (Sigma-Aldrich) for 1 h at 37°C. Primary rat astrocytes were plated onto the Fn-coated wells (2×10^5 cells/well) and allowed to adhere for 24 h. Then, cytokines were added to the cells. After 24 h, 0.5 mM KCC009 or vehicle was added to the cells and a scratch wound was made in each well by using a sterile 10 μ l pipet-tip (Corning). At 0 and 16 h after wound induction, cells were fixed with 4% PFA for 20 min. Cells were washed with PBS and subsequently stained with rhodamine-phalloidin (Invitrogen, 1:300) for 1 h at RT, washed with PBS and embedded in vectashield (Vector Laboratories). Pictures were taken using a Colorview II digital camera (Soft Imaging System, GmbH, Germany). The diameter of the wound (where no cells were present) was measured using Cell*F software (Olympus Soft Imaging Solutions GmbH, Germany). Five random 20x fields per culture condition were captured, and mean wound diameter was assessed in each field and averaged over the five fields.

Bromodeoxyuridine labeling assay

To determine the effect of KCC009 treatment on astrocyte proliferation, primary rat astrocytes were plated onto the Fn-coated wells (2×10^5 cells/well) and allowed to adhere for 24 h. Then, cytokines were added to the cells. After 24 h, 0.5 mM KCC009 or vehicle was added to the cells and a scratch wound was made in each well by using a sterile

10 μ l pipet-tip (Corning). At 1 h after wound induction, 10 μ M bromodeoxyuridine (BrdU; Sigma, 10 μ g/ml) was added for 15 h to label mitotic cells. Cultures were subsequently washed with PBS and fixed with 4% PFA for 20 min, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 10 min and blocked with 3% BSA in PBS for 1 h. To identify BrdU, astrocytes were incubated with mouse anti-BrdU (BD Biosciences, CA, USA, 1:200) in PBS with 1% BSA o/n at 4°C, washed with PBS followed by incubation with donkey anti-mouse Alexa Fluor-488 (Invitrogen, 1:1,000). Cells were washed with PBS and embedded in dapi containing Vectashield (Vector Laboratories). Immunofluorescent staining was visualized using a Leica confocal microscope (Leica Microsystems). In five random 20x fields per culture condition, the number of proliferative cells (%) was calculated as follows: number of BrdU positive cells/number of dapi positive nuclei x 100%.

Visualization of F-actin and vinculin

The effect of KCC009 on focal adhesion formation was visualized by vinculin immunocytochemistry. Cells that were treated with cytokines for 48 h were plated on Fn-coated (2 μ g/cm², Sigma-Aldrich) 8-well chamber slides (Labtek, Nalge Nunc International, 2x10⁴ cells/well) and left o/n at 37°C in serum free medium in the presence of cytokines. The following day, the cells were incubated with 0.5 mM KCC009 in 0.2% DMSO or 0.2% DMSO only (vehicle) in serum free medium for 1 h and subsequently fixed for 20 min with 4% PFA, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 10 min and blocked with 3% BSA in PBS for 1 h. To identify focal adhesions, astrocytes were incubated with mouse anti-vinculin antiserum (Abcam, 1:400) in PBS containing 1% BSA o/n at 4°C, washed with PBS followed by incubation with donkey anti-mouse Alexa Fluor-488 (Invitrogen, 1:1,000). Slides were washed and to identify F-actin cytoskeleton filaments, astrocytes were subsequently stained with rhodamine-phalloidin (Invitrogen, 1:300) for 1 h at RT, washed with PBS and embedded in Vectashield (Vector Laboratories). Immunofluorescent staining was visualized using a Leica confocal microscope (Leica Microsystems). Three independent experiments were performed.

Statistics

Where appropriate, data were analyzed by one-way or two-way ANOVA, followed by a t-test for independent measurements (Fisher's LSD test). The statistical evaluation was carried out by using the NCSS 2007 statistical program (NCSS, East Kaysville, Utah, USA). Error bars represent standard error of the mean (s.e.m.).

Results

TG2 expression in primary rat astrocytes

To detect if TG2 is expressed in primary rat astrocytes, cells were isolated from the cortex of 2 day old rat pups and cultured *in vitro*. TG2 immunoreactivity was present in these cells (Fig. 1A). Since we were interested in the role of TG2 in astrocytes under inflammatory conditions, we studied the effect of various pro-inflammatory cytokines on TG2 expression levels. TG2 mRNA levels were increased after treatment with TNF α , IL-1 β and IFN γ alone and combinations of TNF α and IL-1 β or IFN γ and IL-1 β showed a synergistic increase in TG2 transcript levels (Fig. 1B). TG2 protein levels could be visualized

on western blot (Fig. 1C) and showed an increase after cytokine treatment. The change in TG2 protein levels was quantified using a specific TG2 ELISA. Also at the protein level, all single cytokine treatments resulted in elevated TG2 levels in the astrocytes (Fig. 1D). Moreover, all combined cytokine treatments induced a synergistic increased level of TG2 protein (Fig. 1D). Additionally, TG activity, present in the cell lysates of these astrocytes, is significantly increased after treatment with TNF α and IL-1 β alone, but not after treatment with IFN γ . However, the combined cytokine treatments all enhanced TG activity (Fig. 1E).

Active TG2 on the surface of astrocytes

Using immunocytochemistry on non-fixed cells, we were able to detect TG2 immunoreactivity located on or nearby the surface of primary rat astrocytes (Fig. 2A). To measure semi-quantitative changes in TG2 surface expression after treatment with cytokines, cell surface biotinylation and TG2 immunoprecipitation was performed. This resulted in a clear increase in the amount of TG2 protein present on the surface

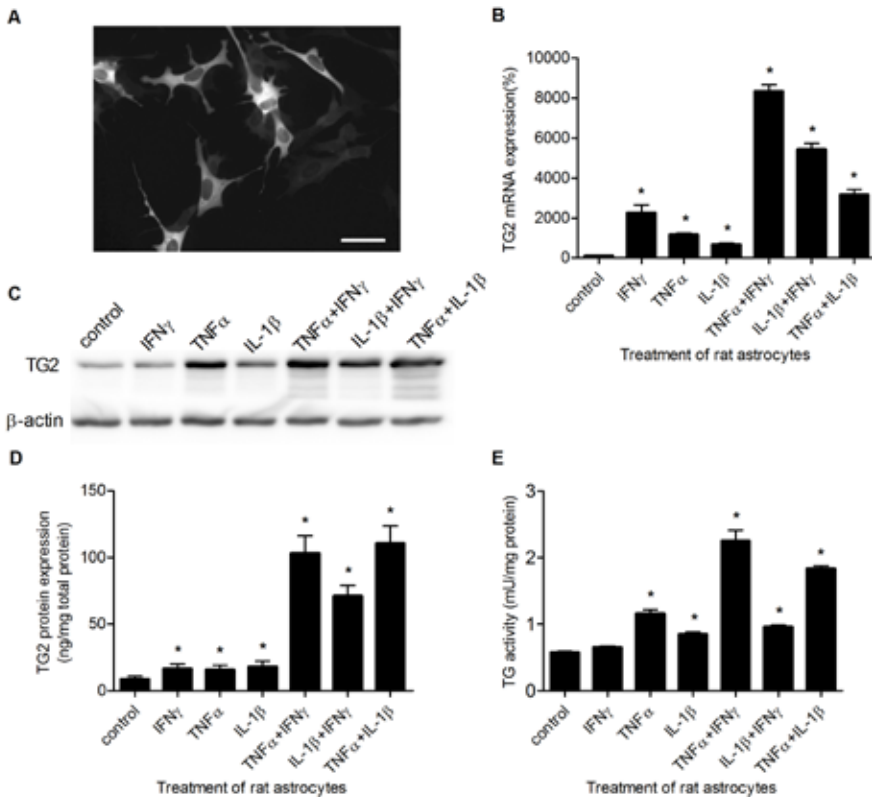


Figure 1: TG2 expression in primary rat astrocytes. **A)** TG2 is expressed in primary rat astrocytes as visualized by immunocytochemical analysis. Scale bar: 50 μ m. **B)** TG2 mRNA expression after treatment with various cytokines alone or in combinations. **C)** TG2 protein expression visualized by western-blot and **D)** measured with ELISA. **E)** TG2 activity levels measured with the TG Covtest TCMA. Data are expressed as mean + s.e.m., n=6 per condition, *P<0.05 compared to control.

of astrocytes, again most dramatically when cells were treated with combinations of cytokines (Fig. 2B). Treatment with single cytokines resulted in an increased TG2 surface expression ranging from ~4x to ~9x the expression in untreated astrocytes whereas treatment with the various combinations of cytokines resulted in expression of ~40x to ~76x compared to untreated cells.

To determine whether this surface associated TG2 is active, we analyzed the incorporation of the competitive amine substrate biotinylated cadaverine onto the surface of astrocytes using FACS analysis (Fig. 2C and Supplementary Fig. 1). The incorporation of biotinylated cadaverine as measured by mean fluorescent intensity was already detectable in untreated cells (control) and clearly increased with 30% or 115% after treatment with IL-1 β or TNF α respectively. Incubation of the astrocytes with the combination of IL-1 β and TNF α resulted in a two-fold increase (200%) in mean fluorescent intensity. The incorporation of biotinylated cadaverine was significantly reduced in a range from ~57% up to 70% after treatment with the specific irreversible TG2 inhibitor KCC009 compared to vehicle-treated control and cytokine-treated astrocytes, respectively (Fig. 2C).

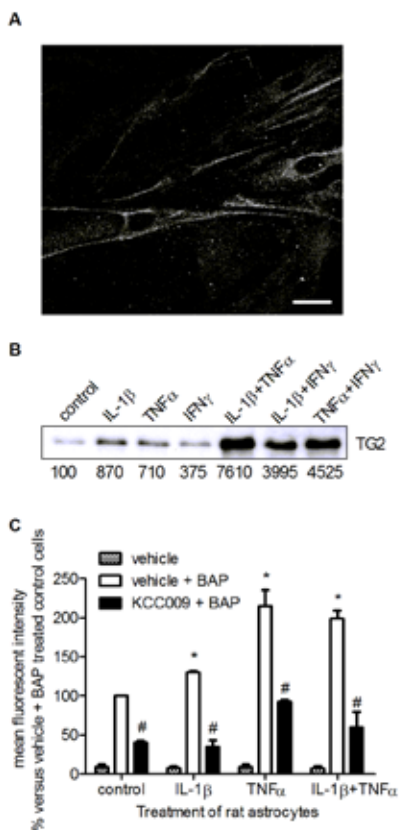


Figure 2: TG2 is expressed on the surface of rat astrocytes. **A)** Life non-permeabilized cells were immunofluorescently stained to detect expression of TG2 on the cell surface of rat astrocytes. Scale bar: 20 μ m **B)** Surface-biotinylated proteins were immunoprecipitated, separated by SDS-PAGE, and blots were stained with a TG2 antibody. Bands were quantified and expressed as % compared to control. **C)** FACS analysis of BAP incorporation on the surface of control rat astrocytes or astrocytes treated with IL-1 β , TNF α or IL-1 β + TNF α in the absence or presence of KCC009. Data represent mean fluorescent intensity \pm s.e.m. from 3 separate experiments. *P<0.05 versus vehicle-treated control cells, #P<0.05 versus vehicle-treated control or matched cytokine-treated.

Astrocyte surface associated TG2 interacts with fibronectin

Since TG2 was present and active on the surface of astrocytes, we questioned whether it interacts with Fn. Therefore, we studied the incorporation of the competitive amine substrate biotinylated cadaverine into Fn.¹⁸⁰ Astrocytes were treated with cytokines for 48 hours and subsequently, cells were plated onto Fn coated wells in serum free medium containing the competitive amine substrate biotinylated cadaverine. After incubation, cells were extracted with deoxycholate containing buffer to eliminate the astrocytes leaving the ECM intact. Any biotinylated cadaverine covalently incorporated by astrocyte-derived TG2 into extracellular Fn was quantified. Compared to untreated astrocytes (control) the amount of incorporated biotinylated cadaverine was significantly increased by 1.5 or 2 times after treatment with $TNF\alpha$ or $IL-1\beta$, respectively. The combination of these cytokines synergistically increased the amount of incorporated cadaverine by approximately 17 times (Fig. 3). To confirm the specificity of biotinylated cadaverine incorporation into Fn by TG2, rat astrocytes were co-incubated with the specific irreversible TG2 inhibitor KCC009. Incubation of cells with the inhibitor resulted in significantly reduced incorporation of biotinylated cadaverine into Fn compared to vehicle-treated astrocytes (Fig. 3).

Inhibition of TG2 activity reduced astrocyte adhesion onto fibronectin

To determine whether TG2 plays a role in adhesion of rat astrocytes onto fibronectin, a quantitative adhesion assay was performed. When astrocytes were cultured in the presence of $IL-1\beta$, $TNF\alpha$ or the combination of these cytokines, astrocyte adhesion onto Fn was increased by 110%, 56% and 64%, respectively (Fig. 4A). Treatment with KCC009, to inhibit TG2 activity, significantly reduced the amount of adherent cells up to 43% compared to vehicle-treated cells (Fig. 4A).

As a control, we checked the effectivity of KCC009 in reducing TG activity in the astrocytes. We already showed in Fig. 1E that cytokine treatment enhanced TG activity. We now confirmed this effect, while co-incubation of the cytokines with KCC009 significantly reduced TG activity in the astrocytes, although it did not return to control levels (Fig. 4B). Of importance was that cytokine and/or KCC009 treatment did not affect astrocyte viability as measured with a propidium iodide assay (Fig. 4C).

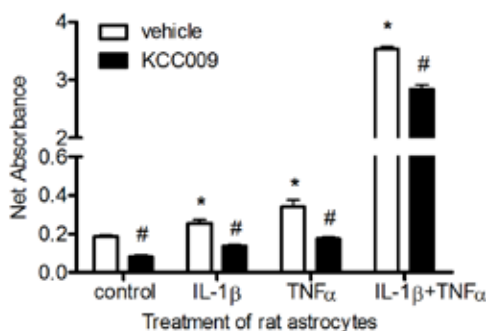


Figure 3: Incorporation of biotinylated cadaverine into Fn by TG2 on the surface of primary rat astrocytes treated with cytokines. Data represent mean values + s.e.m. from a representative experiment, n=5 for each data point. * $P < 0.05$ versus vehicle-treated control cells, # $P < 0.05$ versus vehicle-treated control or matched cytokine-treated cells.

Inhibition of TG2 activity reduced migration of astrocytes across fibronectin

To analyze the potential involvement of TG2 in migration of cytokine stimulated astrocytes, a quantitative migration assay was performed.¹⁷⁴ Astrocytes were plated onto Fn coated wells and, after adherence, a scratch wound was induced to stimulate astrocyte migration in a monolayer. The subsequent migration of untreated astrocytes was compared to cytokine treated cells (Fig. 5A). Initial wound diameter was similar for all conditions tested at $t=0$ (~400 μm). At 16 h after wounding, the diameter reduced to approximately 70 μm in untreated astrocytes (Fig. 5B). Treatment with IL-1 β or the combination of IL-1 β and TNF α resulted in an altered migration capacity of these astrocytes (Fig. 5B). Cells cultured in the presence of TNF α showed an increased migration and reduced the gap to approximately 40 μm (Fig. 5B) To explore whether TG2 activity was affecting cell motility, astrocyte migration was assessed in the presence of KCC009 (Fig. 5A). Treatment with KCC009 in both control and cytokine-treated astrocytes significantly reduced migration compared to vehicle-treated cells (Fig. 5B).

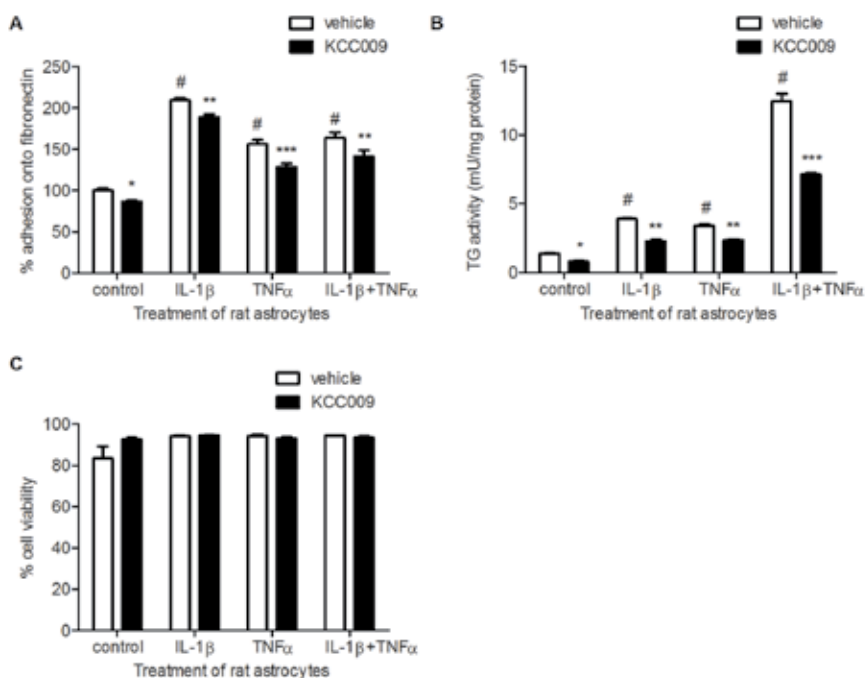


Figure 4: The effect of cytokine treatment and inhibition of TG2 activity on the adhesion capacity of astrocytes onto Fn. **A)** Adhesion of rat astrocytes onto Fn coated wells after treatment with cytokines and subsequent inhibition of TG2 activity. **B)** TG activity after treatment with cytokines and subsequent inhibition of TG2 activity. **C)** Cell viability measured using the propidium iodide assay. Data are expressed as mean + s.e.m., $n=15$ (A) or $n=5$ per condition (B,C), # $P<0.001$ versus vehicle-treated control cells and * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus vehicle-treated control or matched cytokine-treated cells.

To check whether effects on migration were due to altered proliferation of cells, the nuclear uptake of BrdU was measured. Culturing the cells in the presence of IL-1 β , TNF α or the combination of IL-1 β and TNF α significantly increased the nuclear uptake of BrdU by 50%, 140% and 207%, respectively compared to untreated astrocytes (control) (Fig. 5C). In contrast, inhibition of TG2 activity by KCC009 significantly decreased BrdU uptake compared to vehicle-treated control cells (Fig. 5C). It is of interest to note that this decrease resulted in similar reduced levels of astrocyte proliferation independent from the cytokine treatment.

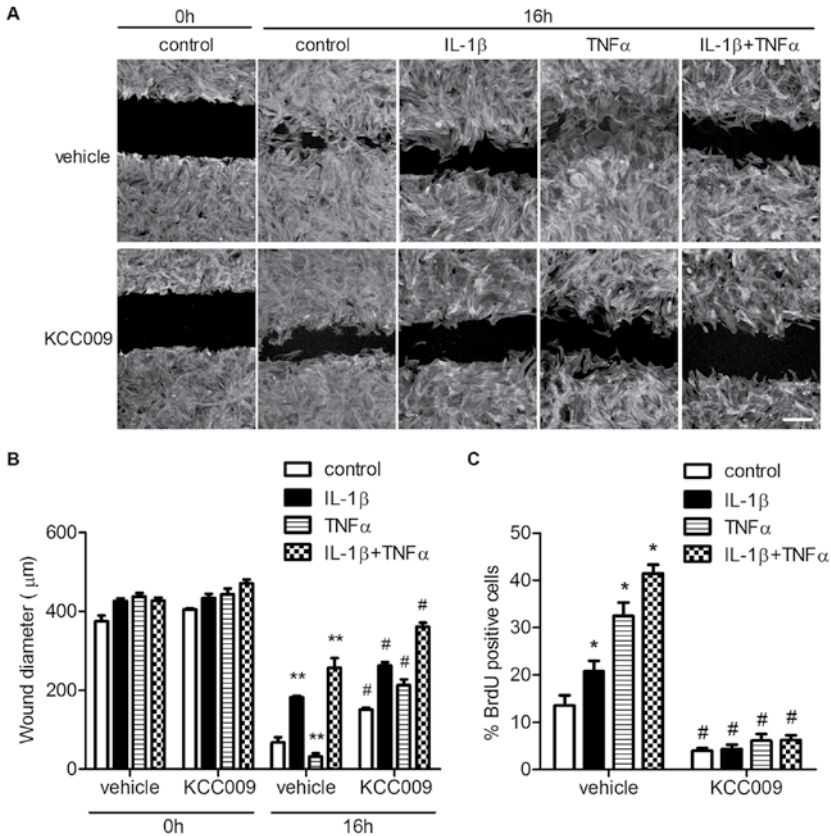


Figure 5: The effect of cytokine treatment and inhibition of TG2 activity on the migration capacity of astrocytes. **A)** A scratch wound was made in an astrocyte confluent cell layer plated onto Fn and astrocytes were allowed to migrate in the presence of vehicle or 0.5 mM KCC009. Cells were fixed after 0 and 16 h and stained with rhodamine-phalloidin to visualize the cells and wound diameter. Scale bar: 200 μ m. **B)** Quantification of wound diameter after migration of rat astrocytes. Astrocytes were allowed to migrate for 16 h and the surface of the wound was quantified using phase-contrast microscopy after 0 and 16 h. **C)** BrdU incorporation after cytokine treatment and subsequent TG2 inhibition. Data represent mean + s.e.m. from a single experiment using 5 measurements per condition/time point and is representative out of 3 separate experiments. * $P < 0.05$ versus vehicle-treated control cells, ** $P < 0.01$ versus vehicle-treated control cells, # $P < 0.01$ versus vehicle-treated control or matched cytokine-treated cells.

Cytokine induced TG2 activity is involved in focal adhesion formation

Using confocal imaging, the effect of cytokine treatment and TG2 inhibition on focal adhesion formation was studied. Cellular adhesion is partly mediated by an intracellular focal adhesion complex that links cell surface integrins to the actin cytoskeleton via integrin receptors and adaptor proteins such as vinculin.⁴⁰⁶ F-actin was visualized using rhodamine-phalloidin and was organized in fine bundles that were orientated along the cellular axis. Untreated astrocytes exhibit vinculin positive focal adhesions at the tip of F-actin stress fibers (Fig. 6A, arrows). When cells were cultured in the presence of IL-1 β , TNF α or the combination of both cytokines, vinculin positive focal adhesions were still present, but less compared to the control (Fig. 6A). Compared to vehicle-treated cells, treatment with KCC009 induced a dramatic redistribution of vinculin, from a focal to a diffuse pattern (Fig. 6A) in both control and cytokine-treated cells.

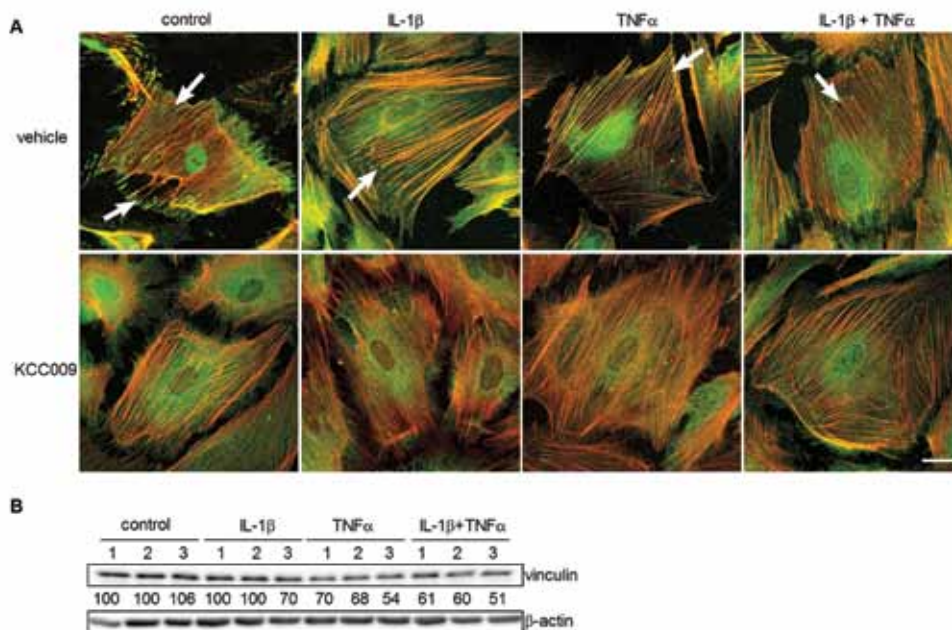


Figure 6: The effect of cytokine treatment and inhibition of TG2 activity on cytoskeletal rearrangements and RhoA activity. **A)** Confocal images of cells stained with rhodamin phalloidin (red) and an antibody against vinculin (green). Scale bar: 10 μ m. Cells were cultured in medium or medium containing IL-1 β , TNF α or the combination of both cytokines for 48 h. Subsequently, cells were treated as follows: 1=untreated control, 2=vehicle, 3=KCC009 and western blot was performed. **B)** Representative western blot of vinculin and β -Actin (n=3) Semi-quantitative analysis of vinculin levels were corrected for β -Actin. The western blot illustrated is representative of three independent experiments.

Western blot analysis was performed to study the relative expression levels of vinculin. Bands were semi-quantified and corrected for β -actin expression levels. Treatment with IL-1 β did not alter the level of vinculin protein. In contrast, vinculin protein levels were reduced after treatment with TNF α or the combination of IL-1 β and TNF α (Fig. 6A,B). Treatment with KCC009 slightly reduced vinculin expression levels in all cytokine-treated cells as compared to control and vehicle-treated cells (Fig. 6B).

Discussion

In the present study, we demonstrate that TG2 expression and activity is present in primary rat astrocytes and increased after treatment with pro-inflammatory cytokines. Moreover, active TG2 is present on the surface of astrocytes and interacts with Fn to mediate astrocyte adhesion and migration. Previous work showed that TG2 is expressed by rat astrocytes and that the pro-inflammatory cytokines IL-1 β and, to a lesser extent, TNF α upregulated expression of TG2.²⁵⁵ However, the functional significance of this increased level of TG2 remained unknown.

Our study provides evidence that pro-inflammatory cytokines, in particular TNF α , IL-1 β and IFN γ or combinations of these cytokines enhance intracellular and cell-surface expression of TG2, even after two days of cytokine treatment. This is of interest as in a recent study we observed the appearance of immunoreactive TG2 in astrocytes in active MS lesions, a chronic neuroinflammatory condition.³⁸⁶ Moreover, in a human astrocytoma cell line surface expression TG2 was detected.³⁸⁶ It should be noted that rat astrocytes treated with a combination of cytokines showed a synergistic increase in TG2 expression and activity. This is in concordance with observations on cytokine-induced synergistic effects in various other cell systems,^{166, 184, 418} indicating that similar down-stream mechanisms are involved. Furthermore, we demonstrated that surface expressed TG2 is active via detection of incorporated biotinylated cadaverine, a substrate for TG's, in astrocytes using FACS analysis. The fact that the observed mean fluorescence intensity could be reduced after inhibition of TG2 activity using the specific inhibitor KCC009, indicated that TG activity on the surface of astrocytes is due to TG2. To determine whether this astrocytic surface TG2 could play a role in ECM rearrangement, we studied the interaction between rat astrocytes and fibronectin, an important ECM protein in MS lesions.^{250, 349, 390} We observed that the enhanced TG2 activity on the cell surface upon cytokine treatment was accompanied by an increased astrocyte adhesion to Fn and that inhibition of TG2 activity, at least partly, reduced astrocyte adhesion. How astrocyte-derived TG2 mediates the adhesion between astrocytes and Fn is uncertain. It could be due to high-affinity binding of surface TG2 to Fn³⁷⁹ via interaction with its gelatin-binding domain.^{10, 305} The interaction between astrocytes and Fn might also be attributed to cell surface TG2 complexed with β -integrins to enhance the affinity for binding to Fn as it has been shown for monocytes¹⁰ and breast cancer cells.²³² Alternatively, TG2 present on the surface of endothelial cells has been demonstrated to contribute, via its cross-linking activity, to the interaction with Fn.²³⁷ In the present study, the increased incorporation of biotinylated cadaverine into Fn which can be inhibited by KCC009 suggests that the active site of TG2, known to be involved in cross-linking activity, is of importance in the interaction between rat astrocytes and Fn. As it is known that TG2 catalysed protein cross-links are stable to proteolytic and mechanical

damage,³⁹² TG2 activity on the surface of astrocytes might facilitate increased stability and resistance to degradation of modified ECM proteins such as fibronectin.

Rat astrocytes treated with IL-1 β or the combination of IL-1 β and TNF α displayed reduced migration on Fn which is in line with previous observations showing that treatment of human astrocytes with IL-1 β attenuated migration on Fn.¹⁷⁴ In contrast, treatment with TNF α resulted in an increased migration capacity of rat astrocytes as observed in other cell types, including cancer cells and mesenchymal stem cells.^{208, 427} Taken together, our data suggest cytokine-specific regulation of astrocyte migration. This is confirmed by observations in neural precursor cells showing that IL-1 β , but not TNF α reduced migration of these cells.³⁶⁰

The role of TG2 in astrocyte migration was determined by inhibiting its activity using KCC009. Reduction of TG2 activity in cytokine-treated rat astrocytes decreased the astrocyte migration capacity compared to matched vehicle-treated cells, suggesting that TG2 activity is important for cell migration, although the effect of IL- β on cell migration was different compared to TNF α . It has been shown that the effect of TG2 on cell migration relates to the interaction of cell surface TG2 with Fn.^{9, 22} We also found a reduction in the interaction of cell-surface associated TG2 with Fn after inhibition of TG2 activity, but this reduced interaction is more likely due to reduced cross-linking of Fn by TG2. This hypothesis is based on observations in fibroblasts where it was shown that inhibition of the active site reduces fibroblast migration whereas TG2 antibodies blocking the interaction with Fn did not affect migration of fibroblasts across Fn.³⁵⁷ The effect of cytokines on astrocyte migration cannot be attributed to altered proliferation, because the treatment combination of IL-1 β and TNF α showed the least migration, but most proliferation of astrocytes. Moreover, inhibition of TG2 activity by KCC009 similarly attenuated astrocyte proliferation independent from cytokine treatment, which is thus not responsible for the cytokine-specific effects observed.

Astrocyte adhesion and migration are multistep processes involving formation and stabilization of focal adhesions which are necessary for cell adhesion. These focal adhesions link the extracellular matrix to the cytoskeleton via integrin receptors and adapter proteins such as vinculin.⁴⁰⁶ Interestingly, treatment with TNF α or the combination of IL- β and TNF α reduced vinculin protein levels, whereas treatment with IL-1 β had no effect. However, focal adhesion formation, as determined by staining for F-actin and vinculin, was, although not quantified, seemingly reduced after treatment with cytokines compared to control. This is in agreement with earlier observations that treatment with cytokines reduced focal adhesion formation in astrocytes.³⁶² Furthermore, it has been shown that TNF α regulates cytoskeletal organization and dispersion of vinculin from focal adhesion sites, resulting in increased migration.¹⁸¹ However, this is the first data showing that TNF α regulates focal adhesion formation resulting in increased adhesion and migration of astrocytes.

After inhibition of TG2 activity, vinculin protein levels were further slightly reduced, suggesting that cytokine-enhanced TG2 does not clearly contribute to the reduction in vinculin protein levels. However, inhibition of TG2 activity by KCC009 does result in a clear intracellular redistribution of vinculin, from a focal to a diffuse cytoplasmic localization was observed. This redistribution might mediate the dissolution of focal adhesions and thereby, most likely, a reduced interaction of astrocytes with the ECM³⁶² and subsequent adhesion and migration. Thus, we suggest that TG2 is an essential factor in regulating the

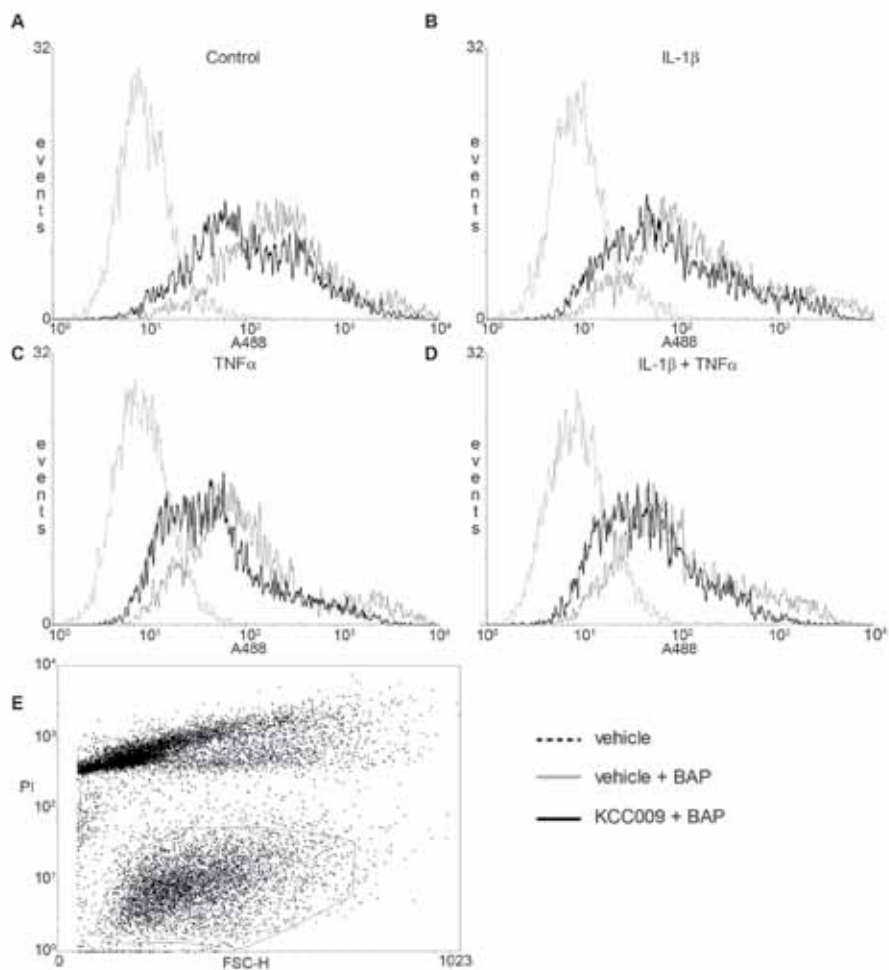
intracellular distribution of vinculin, and thus contributing to focal adhesion formation, necessary for interaction of astrocytes with the ECM, i.e. fibronectin.

In summary, we have shown that TG2 activity present on the surface interacts with the ECM protein Fn. Upon cytokine treatment, TG2 expression and activity on the cell surface is increased, resulting in increased adhesion to and interaction with Fn. Interestingly, treatment with different cytokines resulted in opposite effects on cell migration whereas inhibition of TG2 activity after cytokine treatment reduced astrocyte migration. Thus TG2 is involved in cytokine specific regulation of astrocyte migration. Furthermore, inhibition of TG2 activity resulted in altered focal adhesion formation which is necessary for astrocyte-ECM interaction. Therefore, we put forward that after brain injury or during neuroinflammatory conditions such as MS, TG2 activity on the surface of astrocytes is involved in remodeling and stabilization of the ECM, in particular by interacting with Fn, to facilitate glial scar formation. In support of this, increased expression of TG2 correlated with the level of interstitial kidney scarring *in vivo*.¹⁷⁹ Furthermore, increased expression of TG2 by epithelial cells and its cross-linking function is directly involved in the deposition of collagen and subsequent stabilization of the ECM.¹¹⁰ Thus, astrocyte-derived surface TG2 can play a major role in the interaction with Fn, thereby regulating ECM remodeling and possibly glial scarring.

Acknowledgements

Alvine Pharmaceuticals (San Carlos, USA) is thanked for their generous gift of KCC009. This study was financially supported by the Stichting MS Research (The Netherlands).

Supplementary data



Supplementary Figure 1: FACS plots of BAP incorporation on the surface of rat astrocytes **A)** untreated or treated with **B)** IL-1 β , **C)** TNF α or **D)** IL-1 β + TNF α and subsequent inhibition of TG2 activity. **E)** Determination of living cells which were used for quantification.