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COMPLETE TRANSGLUTAMINASE 2 ABLATION RESULTS IN REDUCED STROKE VOLUMES AND ASTROCYTES THAT EXHIBIT INCREASED SURVIVAL IN RESPONSE TO ISCHEMIA

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Abstract

Transglutaminase 2 (TG2) is a very multifunctional protein that is ubiquitously expressed in the body. It is a Ca²⁺-dependent transamidating enzyme, a GTPase, as well as a scaffolding protein. TG2 is the predominant form of transglutaminase expressed in the mammalian nervous system. Previously, it was shown that TG2 can affect both cell death and cell survival mechanisms depending on the cell type and the stressor. In the case of ischemic stress, TG2 was previously shown to play a protective role in the models used. For example in hTG2 transgenic mice, where TG2 is overexpressed only in neurons, middle cerebral artery ligation (MCAL) resulted in smaller infarct volumes compared to wild type mice. In this study TG2 knock out mice were used to determine how endogenous TG2 affected stroke volumes. Intriguingly, infarct volumes in TG2 knock out mice were significantly smaller compared to wild type mice. As expected, primary neurons isolated from TG2 knock out mice showed decreased viability in response to oxygen-glucose deprivation. However, primary astrocytes that were isolated from TG2 knock out mice were resistant to oxygen-glucose deprivation *in situ*. Both wild type and knock out neurons were protected against oxygen glucose deprivation when they were co-cultured with astrocytes from TG2 knockout mice. Therefore, the decreased stroke volumes observed in TG2 knock out mice after MCAL, can be correlated with the protective effects of TG2 knock out in astrocytes in response to oxygen glucose deprivation *in situ*. These findings suggest that neuron-astrocyte crosstalk plays a significant role in mediating ischemic cell death and that TG2 differentially impacts cell survival depending on cell context.

Keywords

Transglutaminase 2; stroke; ischemia; hypoxia; middle cerebral artery ligation; cell death; neuron; astrocyte

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Introduction

Stroke remains as a leading cause of death in the United States in part due to the lack of effective therapeutics for most patients (Cramer, 2011). The complexity of intracellular signaling mechanisms that are triggered in an ischemic event, as well as the variety of cell types that are affected in the brain, have made the search for effective treatments a daunting task. However it is becoming increasingly evident that subsequent to stroke the coordinated response of all the cells in the brain, including neurons, glia and vascular endothelial cells, plays a significant role in the final outcome (Yenari and Lee, 2011). Therefore it is critically important to understand how each cell type responds to ischemic stress and in turn how this impacts the survival of the other cells in the affected area of the brain.

Neuron-astrocyte interactions are especially important when it comes to neurodegenerative pathologies, as astrocytes have many functions that support the viability of neurons such as buffering the extracellular space, providing substrates to neurons and interchanging glutamate for glutamine (Verkhratsky and Toescu, 2006). Neurons and astrocytes respond differently to ischemic stress, in part due to differences in their cellular metabolism. Astrocytes are more dependent on glycolytic metabolism and they possess glycogen stores which makes them less susceptible to ischemia. In contrast, neurons are highly dependent on oxidative metabolism and thus are more sensitive to oxygen-glucose deprivation (Escartin et al., 2006). The support of neurons by astrocytes is very important in ischemic conditions. Deficits in astrocytes function can negatively impact stroke size and recovery. If the astrocytic functions such as extracellular buffering or glutamate uptake are compromised, infarct size might increase due to an increase loss of neurons (Rossi et al., 2007). Additionally, astrocytes express neuroprotective factors such as erythropoietin (EPO) (Diaz et al., 2005; Liu et al., 2006), vascular endothelial growth factor (VEGF) (Rosenstein and Krum, 2004) and adenosine (Hamilton and Attwell, 2010; Schubert et al., 1997) that facilitate neuronal survival in ischemia. On the other hand, astrocytes also release neurotoxic factors such as nitric oxide (NO) which can potentiate neuronal cell death (Hu et al., 1997; Saha and Pahan, 2006). Therefore, the role of astrocytes in ischemic neuronal death is a crucial factor in regulating the size of an infarct.

Transglutaminase 2 (TG2) belongs to a family of enzymes that catalyze Ca^{2+} dependent transamidation reactions which can form isopeptide bonds between the γ -carboxamide group of a peptide bound glutamine residue and ϵ -amino group of a lysine residue (Griffin et al., 2002). TG2 can either facilitate or attenuate cell survival depending on the stressor, as well as the cell type (Gundemir and Johnson, 2009). In neurons, TG2 was found to be protective against ischemic cell death both in vitro and in vivo (Filiano et al., 2008; Filiano et al., 2010). Although TG2 is present in astrocytes and is upregulated in response to different stressors (Caccamo et al., 2005; Campisi et al., 2008; Campisi et al., 2004; Monsonego et al., 1997), how TG2 affects survival of astrocytes in response to ischemia has not been investigated. Astrocytes likely play a critical role in the pathogenesis of ischemia-induced neuronal cell death (Takano et al., 2009); therefore it is important to understand how TG2 impacts cell death processes in both neurons and astrocytes.

TG2 protein levels, as well as the transamidation activity were increased in the hippocampus after ischemia-reperfusion injury in gerbils (Ientile et al., 2004). Further, we have shown that middle cerebral artery ligation (MCAL)-induced infarct volumes are significantly smaller in mice that selectively overexpress human TG2 in neurons (hTG2 mice) compared to wild type mice (Filiano et al., 2010). TG2 was also protective against oxygen-glucose deprivation (OGD) when overexpressed in rat primary neurons (Filiano et al., 2008). Here, we used a complete embryonic TG2 knock out (TG2^{-/-}) mouse (Nanda et al., 2001) to study the effect of TG2 ablation in stroke injury. Interestingly, stroke volumes in TG2^{-/-} mice were

significantly smaller 24 h after MCAL when compared with wild type mice. This protection was not due to differences in HIF-1 regulated gene expression in TG2^{-/-} mice brain after stroke. The primary neurons isolated from TG2^{-/-} mice were actually less viable than neurons from wild type mice following OGD. However, primary astrocytes isolated from TG2^{-/-} mice were significantly more resistant to OGD-induced cell death compared to astrocytes from wild type mice. Both wild type and TG2^{-/-} mouse primary neurons were protected against OGD when they were co-cultured with astrocytes from TG2^{-/-} mice. Therefore, we can conclude that the increased survival of astrocytes from TG2^{-/-} mice after OGD, and the fact that TG2^{-/-} astrocytes protect neurons against OGD, could contribute to smaller infarct volumes in TG2^{-/-} mouse brain after MCAL. The results of these studies clearly demonstrate that cell context plays a significant role in determining whether TG2 facilitates or ameliorates ischemic cell death.

Materials and Methods

TG2^{-/-} mice

TG2^{-/-} mice was generously provided by Dr. Robert M. Graham at University of New South Wales in Australia. Briefly, the Cre/*loxP* site-specific recombination system of bacteriophage P1 was used to develop mouse lines in which *Tgm2* can either be ubiquitously inactivated or selectively inactivated in specific tissues. A gene-targeting construct in which *loxP* sites were inserted which flank exons 6–8 and it was transfected into W9.5 mouse embryonic stem cells. The homologous recombination events were confirmed by Southern blot analysis. Chimeric mice were back-crossed with C57BL/6 mice to obtain heterozygous floxed mice. TG2^{-/-} mice were generated by crossing heterozygous or homozygous floxed mice with transgenic mice expressing Cre recombinase in the germline under the control of the human cytomegalovirus minimal promoter (Nanda et al., 2001).

Cerebral Macrovascular Morphology

Mice were deeply anesthetized with 3% isoflurane and 20% oxygen gas mixture and transcardially perfused with pre-warmed (37°C) saline solution containing 20% (w/v) gelatin and 0.2% (w/v) bromophenol blue. Brains were removed immediately and the Circle of Willis was photographed with a camera attached to a dissecting scope. For determination of the localization of middle cerebral artery, the angle between the middle cerebral artery and anterior cerebral artery was measured (Filiano et al., 2010; Maeda et al., 1998).

Cerebral Microvascular Morphology

Mice were deeply anesthetized with 3% isoflurane, 20% oxygen gas mixture and transcardially perfused first with phosphate buffer saline and subsequently with phosphate buffer saline (PBS) containing 4% paraformaldehyde. Brains were removed; paraffin embedded, and 6µm coronal slices were collected every 600µm between +2.68 and -3.80 from bregma. The slices were deparaffinized with HistoClear solution (Electron Microscopy Science) and the tissue was rehydrated with isopropanol followed by incubating with liberating antibody solution (L.A.B. Polysciences) for antigen retrieval. The sections were incubated with fluorescein labeled Lycopersicon Esculentum (Tomato) Lectin (250 µg/ml in PBS) for 1 h at 37 °C. Slides were then washed, mounted, and imaged with an Axiovert inverted microscope at 40× with attached AxioCam-XMR camera (Zeiss) (Filiano et al., 2010). The total number of objects (vessels) was counted per cortical area by using Image J software (<http://rsbweb.nih.gov/ij/>). Data is represented as total number of vessels per mouse brain cortex by taking summation of number of particles counted in each brain slice.

Middle Cerebral Artery Ligation

All mice used in this study were 10- to 12-week-old males approximately 25 g in weight. Mice were deeply anesthetized with 3% isoflurane and 20% oxygen gas mixture. Using a dissecting microscope, a small 5-mm vertical skin incision was cut between the left eye and ear to expose the skull. The temporal muscle was retracted and a small hole was made on the skull with an 18 gauge needle at the junction of the zygomatic arch and squamosal bone. The middle cerebral artery was exposed by further craniotomy and was cauterized distal of the lenticulostriate arteries. The incision was closed with sterile 5-0 nylon monofilament sutures. Buprenorphine was used at 0.05 mg/kg, sc prior to surgery, and then every 5 h for the next 24 h post-surgery. Body temperature was maintained at 37 °C with a heating pad during surgery (approx. 30 min) and recovery (Colak et al., 2011a).

Twenty-four hours after the surgery, the mice was transcardially perfused with a 4% 2,3,5 Triphenyltetrazolium chloride (TTC) (w/v) in PBS for 15 min and then with a 4% paraformaldehyde in phosphate buffer saline for 10 min by using a mini peristaltic pump at medium flow rate. The brain was removed and placed into the 4% paraformaldehyde solution overnight. The brain was sectioned into 1mm thick slices by using a sectioning block. The slices were photographed by using a digital camera connected to the dissecting microscope. The stroke area was calculated by subtracting the non-infarcted area of the ipsilateral site from the total area of the contralateral site using Image J Software (<http://rsbweb.nih.gov/ij/>). The stroke volume was calculated by stacking the stroke area of the slices (Lin et al., 1993).

Quantitative Real-time PCR (Q-PCR)

RNA was collected by using Trizol reagent (Invitrogen) according to manufacturer's instructions. After isopropanol precipitation, RNA was treated with DNase I (Invitrogen) to eliminate any contamination of genomic DNA. RNA concentrations were measured and cDNAs were made by using SuperScript III Reverse Transcriptase according to the manufacturer's instructions. cDNAs were diluted 1:10 and 2 µl per well was used in a 96-well plate format for Q-PCR with TG1 (Mm_00498375), TG2 (Mm_00436980), TG3 (Mm_00436999), TG5 (Mm_00551325), TG6 (Mm_00624922), Enolase1 (Mm_01619597), Vegfa (Mm_01281449), Noxa (Mm_00451763) pre-made primer sets (Applied Biosystems). Data was normalized to actin (Mm_02619580; Applied Biosystems). Amplification was performed using an ABI Prism 7300 Sequence Detection System (Applied Biosystems) at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of a 15-s step at 95 °C and a 1-min step at 60 °C. Data were analyzed using the $\Delta\Delta C_t$ method as previously described (User Bulletin No. 2, Applied Biosystems).

Mouse Primary Neuronal Cultures

The wild type and TG2^{-/-} mouse neurons were prepared as described in (Vangeison et al., 2008). Mouse brains were collected from day 14–15 embryos. Meninges were removed and cortices were digested in trypsin for 20 min. Cortices were washed with minimum essential media and briefly triturated. Cells were plated as 4×10^4 cells/ well in 12 well plates in neurobasal media (2% B27, 0.5mM glutamine, 25µM glutamic acid). Cultures were composed of >95% neurons (data not shown).

Mouse Primary Astrocyte Cultures

Astrocyte cultures were prepared as described in (Rempe et al., 2007) with slight modifications. Mouse brains were removed from P0 mouse pups. The cortex was dissected and placed into MEM (Invitrogen) supplemented with 6 g/L glucose, 1 mM sodium pyruvate, 10% FBS. Cortices were dissociated with brief trituration. Astrocyte cultures were

composed of >95% astrocytes (data not shown). Culture were maintained until confluent in a humidified incubator at 5 % CO₂ at 37 °C and split onto 12 well plates for experiments. Cultures were composed of >95% astrocytes (data not shown).

For viral transduction, TG2^{-/-} mouse astrocytes were plated at 70 % confluency on 12 well plates in growth media and after 24 h viral particles were added directly to the culture media. Cells were incubated overnight in a humidified incubator at 5 % CO₂ at 37°C. Twenty four hours after transduction, 0.5mM Ara C was added to prevent further cell proliferation and cells were kept for 5–7 days to allow for expression of the transduced constructs. The media was replaced with fresh media every 3 days. Expression of constructs was confirmed by western blot analysis.

Mouse Neuron-Astrocyte Co-cultures

Astrocyte culture was prepared as described above and grown until they reach 70% confluency after which 0.5 mM Ara C was added to prevent microglial proliferation. Neurons were prepared as described above and plated directly on top of the astrocytes. Co-cultures were grown until DIV 10 and their media was half replaced with fresh neuronal media every 4 days. Treatments were performed on DIV11.

Lentivirus Production

Lentivirus production and transduction were performed as described in (Colak et al., 2011b). Wild type TG2 was cloned into FIGB lentiviral vector. TG2 expression was driven by a CMV promoter, with GFP downstream under the control of IRES (Lois et al., 2002). The lentiviral vector backbone was a generous gift from Dr. C. Proschel at the University of Rochester. Viral particles were made in HEK 293T cells (ATCC, cat. no. CRL-11268) by cotransfection of the lentiviral vector, pMD2.G (VSVG-envelope) and psPAX2 (packaging components) plasmids (Addgene.org). Viral particles were concentrated by centrifugation of cell media at >50,000 × g for 2 h at 4 °C. The pellet was resuspended in 1 % BSA in sterile PBS.

Immunoblotting

Cell lysates were blotted as described (Milakovic et al., 2004) with modifications. The blots were probed overnight at 4 °C with anti-TG2 antibody TG100 (Neomarkers) (1:2000) or anti-actin (Chemicon Inc.) (1:5000) antibodies in 5 % non-fat dry milk in TBST. The membranes were rinsed three times for 30 min with TBST and incubated with HRP-conjugated secondary antibody (1:2000) in 5 % non-fat dry milk in TBST for 2 h at room temperature. Lastly, the blots were rinsed three times for 1 h with TBST and developed with a chemiluminescence solution as previously described (Thorpe and Kricka, 1986).

Cell Treatment Paradigm

Neuronal cultures were transferred to glucose free neurobasal media (Invitrogen) supplemented with 0.1mM glucose for 2 h in a humidified hypoxic chamber (Coy Laboratory Products) at 5 % CO₂, 0.2% O₂ at 37 °C for OGD treatment. After the treatment, the cultures were reoxygenated for 24 h in humidified incubator at 5% CO₂ at 37 °C. Resazurin assay was performed to detect cell viability.

Non-transduced or transduced astrocyte cultures were transferred to artificial cerebrospinal fluid (ACSF) buffer (130mM NaCl, 3mM KCl, 1.2mM KH₂PO₄, 20mM NaHCO₃, 3mM HEPES, 1.3mM MgSO₄, 2.4mM CaCl₂, 0.1mM glucose) for 12 h in humidified hypoxic chamber at 5 % CO₂, 0.2% O₂ at 37 °C for OGD treatment. After treatment, the cultures were reoxygenated for 7h in humidified incubator at 5% CO₂ at 37 °C. Resazurin assay was performed to detect cell viability (see below).

Neuron-astrocyte co-cultures were transferred to glucose free neurobasal media (supplemented with 0.1mM glucose) for 4 h in humidified hypoxic chamber at 5 % CO₂, 0.2% O₂ at 37 °C for OGD treatment. After the treatment, the cultures were reoxygenated for 24 h in a humidified incubator at 5% CO₂ at 37 °C. The resazurin assay was performed to detect cell viability. This treatment does not cause any cell death on astrocytes (data not shown); therefore the loss of cell viability is due to neuronal cell death (Vangeison et al., 2008). The co-cultures were treated with 4 h OGD which is longer than the treatment of neuronal cultures alone (2 h), because that neurons are less susceptible to OGD when they are in co-culture system.

During all the OGD treatments, control samples were kept in normoxia conditions in the presence of 25mM glucose in a humidified incubator at ambient oxygen, 5 % CO₂ at 37 °C. There was no significant effect of glucose deprivation alone (0.1mM glucose) on the cell viability of the samples kept under normoxia conditions.

Resazurin Cell Viability Assay

Resazurin was added directly to the culture media to a final concentration of 25µg/ml. After 2 h, fluorescent readings were taken at excitation 530 nm and emission 590 nm by using a plate reader (BioTek Synergy HT Multi-Detection Microplate Reader). The fluorescence intensity is proportional to the number of viable cells. Cell viability is represented as percent fluorescent reading of OGD-treated samples compared to normoxic control samples.

Statistics

All data were expressed as mean $\pm$ SEM and were plotted using Graphpad Instat software. Unpaired t test, paired t test, ANOVA and Tukey's test were used for statistical analysis. Results are shown as mean $\pm$ standard error, * p < 0.05, ** p < 0.01, *** p < 0.001.

Results

The levels of the transglutaminases in TG2^{-/-} mouse brain

TG2^{-/-} mice show no deficiencies in embryonic development and no evident abnormalities under normal physiological conditions (De Laurenzi and Melino, 2001; Nanda et al., 2001). The fact that there are eight active and one inactive transglutaminases expressed in mammalian systems (Griffin et al., 2002) could lead to compensatory upregulation of another transglutaminase in TG2^{-/-} mice. In order to further characterize the TG2^{-/-} mice, mRNA was collected from adult wild type and TG2^{-/-} mouse brains and quantitative RT-PCR was performed to identify levels of different transglutaminases (TG1, TG2, TG3, TG5, and TG6) (Figure 1). In both wild type and TG2^{-/-} mice TG1 and TG3 were detectable, and as expected TG2 transcript was readily detected in the wild type mouse brain. Some very minimal TG2 transcript level was also detected in the TG2^{-/-} mice. The primers used for the TG2 transcript amplify a region spanning exons 3–4. The TG2^{-/-} mice were created by Cre-deleting exons 6–8 (Nanda et al., 2001). Therefore the primers are likely amplifying exons 3–4 on some residual short, non-functional transcripts that are not immediately degraded after transcription (Bailey et al., 2004). There was no difference between the TG3 levels in wild type and TG2^{-/-} mice. The levels of TG5 and TG6 were undetectable in wild type and TG2^{-/-} mouse brain (data not shown). A recent study showed that TG1, TG2, TG3, TG5, TG6 and TG7 are present in BALB/c mouse brain (Thomas et al., 2011). Given these results we repeated the quantitative RT-PCR analysis using the primers that were described for TG5, TG6 and TG7 in (Thomas et al., 2011). However, were still unable to detect TG5 and TG6 in C57/BL6 mouse brain (data not shown). The apparent discrepancy could possibly be due to the difference between the mouse strains. Interestingly, we were able to detect the transcript for TG7 using the primers from (Thomas et al., 2011), but there

was no difference in TG7 transcript levels between wild type and TG2^{-/-} mouse brain (data not shown). These data indicate that there is likely no upregulation of other transglutaminases to compensate for the absence of TG2 in TG2^{-/-} mouse brain.

Macrovascular and microvascular structure of TG2^{-/-} mouse brain

TG2 is expressed by a variety of cell types in the brain including the endothelial cells where it is involved in extracellular matrix interactions and cellular migration (Balklava et al., 2002; Zemskov et al., 2006). In a recent study, knockdown of TG2 by siRNA in endothelial cells decreased proliferation, cell adhesion and increased cell death (Nadalutti et al., 2011). Considering that TG2 is ablated in all the cells in TG2^{-/-} mice, we performed macrovasculature and microvasculature analysis to determine if there were any changes in the brain vasculature due to the absence of TG2. Any morphological change in the location of middle cerebral artery or vascular density in the cortex might affect the blood flow in the infarct region due to changes in the collateral blood flow to that area. These changes could affect the size of the infarct volume. In our macrovasculature analysis we examined the localization of the middle cerebral artery at its branching point on the Circle of Willis. The angle between the middle cerebral artery and a straight line drawn through Circle of Willis (Figure 2A) was measured (Figure 2B) (Maeda et al., 1998). We found no difference between wild type and TG2^{-/-} mice regarding the localization of the middle cerebral artery.

For the microvasculature analysis brain sections were stained with FITC-lectin to label the blood vessels. The total number of particles (vessels) per cortical area was counted to represent the vascular density (Figure 2C) (Baranova et al., 2007; Hohenstein et al., 2005). There was no difference between the total number of vessels counted between the wild type and TG2^{-/-} mouse brains (Figure 2D).

TG2^{-/-} mice are protected against middle cerebral artery ligation (MCAL)

Previously in our laboratory we found that TG2 protects neurons against ischemic-induced cell death. Overexpression of hTG2 selectively in neurons resulted in significantly smaller infarct volume compared to wild type after MCAL surgery (Filiano et al., 2010). In order to test the effect of TG2 ablation on the ischemic response, MCAL surgery was performed on wild type and TG2^{-/-} mice. Twenty-four hours after the surgery stroke volumes were quantified. Interestingly, the stroke volumes in the TG2^{-/-} mice were significantly smaller (~36%) than in wild type mice (Figure 3). However, in contrast to the hTG2 mice where the TG2 is overexpressed selectively in neurons, in the TG2^{-/-} mice TG2 is deleted in all cells. Given that TG2 modulates cell death processes in a context dependent manner, we investigated further what factors may be contributing to the decreased stroke volume in the TG2^{-/-} mice.

Relative TG3 expression is not changed in TG2^{-/-} mice brain after stroke

Previously, it was shown that TG2 mRNA level was upregulated 5 h after MCAL surgery in wild type mouse brain (Filiano et al., 2010). Since TG3 was detectable in TG2^{-/-} mice cortex (Figure 1), we tested whether TG3 was upregulated in TG2^{-/-} mouse brain following stroke and thus is a possible prosurvival signal. Wild type and TG2^{-/-} mice cortices were collected 5 h and 24 h after MCAL surgery. Relative TG3 expression level in the cortex was determined in both ipsilateral and contralateral sides by using quantitative RT-PCR. There was no change in mRNA levels of TG3 in TG2^{-/-} mouse cortex compared to wild type at 5 h (Figure 4A) and 24 h (Figure 4B) after MCAL.

Relative expressions of enolase 1, VEGF and Noxa are not changed in TG2^{-/-} mice after stroke

The protective role of TG2 in the hTG2 mouse model is possibly due in part to the changes in HIF-1 responsive gene expression profile after MCAL. Previously, we found that Noxa expression was significantly attenuated in hTG2 mouse brain compared to wild type mice at 5 h after MCAL surgery (Filiano et al., 2010). Noxa is a HIF-1 regulated proapoptotic gene that is upregulated after hypoxia (Kim et al., 2004). Therefore, downregulation of Noxa expression might contribute to the protection against ischemia in hTG2 mice. On the other hand, TG2 knock down in HEK293 cells resulted in increased expression of enolase 1 and vascular endothelial growth factor (VEGF) genes in response to hypoxia (Gundemir and Johnson, unpublished data). In order to test if HIF-1 responsive gene expression is changed in TG2^{-/-} mice after stroke, mRNA was collected from both wild type and TG^{-/-} mouse cortices at 5h and 24 h after MCAL and quantitative RT-PCR was performed to detect the levels of HIF-1 regulated pro-survival and pro-cell death gene expression. For this purpose, enolase 1 and VEGF were chosen as pro-survival genes, and Noxa was chosen as a pro-cell death gene. There was no difference in the fold mRNA expression of enolase 1, VEGF or Noxa between wild type and TG2^{-/-} mice brain at 5h (Figure 5A) and 24 h (Figure 5B) after MCAL. Therefore, the protection that we observed in TG2^{-/-} mice against stroke is not associated with expression level changes of enolase 1, VEGF or Noxa genes.

TG2^{-/-} mouse primary neurons are more susceptible to OGD

TG2 was shown to be protective in rat primary neurons in response to OGD. In order to determine the effect of TG2 ablation in neurons after ischemia, primary cortical neurons were cultured from wild type and TG^{-/-} mice. The cultures were exposed to an OGD-reoxygenation treatment paradigm (Vangeison et al., 2008) and cell viability was subsequently determined by using the resazurin assay. TG2^{-/-} mouse primary neurons showed significantly lower (~12%) viability compared to wild type mouse neurons after OGD (Figure 6A). This suggests that TG2 ablation in neurons potentiates OGD induced cell death. Therefore, the reduction in stroke volume in the TG2^{-/-} mice was not due to the absence of TG2 in the neurons, which supports our previously published results (Filiano et al., 2010).

TG2^{-/-} mouse primary astrocytes are protected against OGD

We next examined the sensitivity of TG2^{-/-} and wild type astrocytes to ischemic-induced cell death. Astrocyte cultures were exposed to OGD-reoxygenation treatment and cell viability was determined by using resazurin assay. TG2^{-/-} mouse astrocytes showed significantly greater viability (~25%) compared to wild type astrocytes after the treatment (Figure 6B). There was no difference in the growth rate of the TG2^{-/-} and wild type astrocytes (data not shown) and therefore the difference in the viability of the astrocytes was not due to a difference in the confluence of the cultures. This suggests that TG2 ablation in astrocytes protects them against OGD-induced cell death. These findings suggest that the reduction in stroke volume in the TG2^{-/-} mice might be due in part to the increased survival of TG2^{-/-} astrocytes.

Mouse primary neurons are protected against OGD when they are co-cultured with TG2^{-/-} astrocytes

Astrocytes support the metabolic demands of neurons in the brain. Therefore, we next determined how astrocytes from wild type and TG2^{-/-} mice affect neuronal viability subsequent to the OGD-reoxygenation treatment. Wild type and TG2^{-/-} neurons were co-cultured with wild type or TG2^{-/-} astrocytes subsequent to an OGD-reoxygenation treatment paradigm and cell viability determination. The treatment paradigm used did not

affect the viability of astrocytes alone (data not shown). Therefore, the loss of viability in the co-cultures was a result of neuronal cell death in response to the treatment (Vangeison et al., 2008). Based on the resazurin assay, both wild type and TG2^{-/-} mouse neurons showed greater viability (~10%) after OGD-reoxygenation when they were co-cultured with TG2^{-/-} astrocytes which was significantly higher than the viability of wild type neurons co-cultured with wild type astrocytes (Figure 6C). This suggests that TG2^{-/-} astrocytes can increase neuronal viability after OGD-reoxygenation, independent of the type of the neurons. Overall these data suggest that the decreases in stroke volume in TG2^{-/-} mice may be due in part to increased viability of astrocytes, as well as increased neuronal survival that results from a possible support mechanism provided by the TG2^{-/-} astrocytes.

The protection of TG2^{-/-} primary astrocytes against OGD is primarily due to absence of TG2

We next transduced TG2^{-/-} primary astrocytes with control and TG2 expressor lentivirus (TG2 addback) in order to test if the TG2 ablation directly contributes to the increased survival in TG2^{-/-} primary astrocytes after OGD. The expression of exogenous TG2 in the astrocytes was confirmed by immunoblot analyses (Figure 7A). Adding back TG2 to the TG2^{-/-} astrocytes decreased viability following OGD treatment (Figure 7B). This data suggest that endogenous TG2 in astrocytes negatively affects cell viability in response to OGD.

Discussion

It is becoming increasingly apparent that the interplay between astrocytes and neurons is crucial in determining infarct size following a stroke (Rossi et al., 2007). Neurons are more sensitive to ischemic stress compared to astrocytes due to their dependency on oxidative phosphorylation and their susceptibility to glutamate toxicity (Panickar and Norenberg, 2005). The resistance of astrocytes to OGD is in part due to their glycolytic metabolism as well as their glycogen stores (Silver et al., 1997). On the other hand, astrocytes are more susceptible to acidosis due to the high production of lactic acid from increased glycolysis during ischemia (Giffard et al., 1990). Therefore, the factors that determine the ischemic cell death in different cells of the penumbra can be very complex.

In this study, we observed that stroke volumes were significantly reduced in TG2^{-/-} mice compared to wild type mice. Initially this result was surprising since overexpression of human TG2 selectively in neurons (hTG2 mice) also significantly decreased stroke volumes (Filiano et al., 2010). This decrease in stroke volume was not due to compensatory upregulation of other transglutaminases or changes in the vascular structure or density in the TG2^{-/-} mouse brain. In contrast to the hTG2 mouse model, no differences in the expression levels of enolase 1, VEGF and Noxa were observed in TG2^{-/-} mouse brain after MCAL. There was no difference between HIF-1 protein levels in wild type and TG2^{-/-} mouse brains 24 h after MCAL (data not shown). Therefore the difference in stroke volume is likely not due to difference in HIF-1 activation. In hTG2 mice TG2 is selectively overexpressed in neurons but in the TG2^{-/-} mouse TG2 is ablated in all the cells. Given that TG2 can play opposite roles in response to a stressor depending on the cell context, the contradiction in our results could be due to differential role of TG2 in neurons vs other brain cell type such as glia. Therefore, these two mouse models are not really comparable with each other.

TG2^{-/-} mice have normal embryonic development and show no evident abnormalities under normal physiological conditions (De Laurenzi and Melino, 2001). However, the absence of TG2 does result in alterations in a variety of different cellular processes. For example, TG2^{-/-} primary fibroblasts shows a decrease in adhesion and impairment of wound healing,

as well as altered cytoskeleton dynamics (Verderio et al., 2004). $TG2^{-/-}$ mice also are resistant to experimental endotoxic shock induced by lipopolysaccharide (LPS) by increased neutrophil phagocytic ability with attenuated neutrophil extravasation (Falasca et al., 2008). These and other studies (for a review see (Iismaa et al., 2009)) clearly demonstrate that TG2 plays a modulatory role in many signaling systems in different cell types.

In our study, in order to delineate the possible protective role of TG2 ablation in $TG2^{-/-}$ mice, primary neurons and astrocytes, as well as neuron-astrocyte co-cultures were treated with OGD-reoxygenation paradigm. Interestingly, $TG2^{-/-}$ neurons were more susceptible to OGD-reoxygenation, which is in agreement with our previous studies showing the TG2 overexpression protects neurons against cell death induced by OGD-reoxygenation (Filiano et al., 2010). In contrast, $TG2^{-/-}$ astrocytes were significantly more resistant to cell death induced by OGD-reoxygenation when compared with astrocytes from wild type mice. When we cultured wild type or $TG2^{-/-}$ neurons with $TG2^{-/-}$ astrocytes, they were also protected against OGD-reoxygenation treatment. These results suggest that $TG2^{-/-}$ astrocytes are not only more resistant to ischemia-induced cell death, but they also support the viability of neurons, both of which likely contribute to the decrease in stroke volume in $TG2^{-/-}$ mice.

Intercellular interactions play a significant role in the complexity of the penumbral cell death mechanisms in the ischemic brain. Astrocytes support the viability of neurons, but in ischemic conditions can also contribute to the demise of neurons. Astrocytes are involved in maintaining ion homeostasis (Silver et al., 1997), as well as glutamate uptake, both of which can be impaired by ischemia and result in excitotoxicity (Phillis and O'Regan, 1996). During oxygen-glucose deprivation, astrocytes can generate energy through glycolysis which produces high amounts of lactic acid and lead to acidosis (Bondarenko et al., 2005; Folbergrova et al., 1997). Loss of ion homeostasis, glutamate uptake and increased acidosis will cause astrocytes to swell leading to edema (Gunnarson et al., 2008) and cause glutamate release due to activation of volume regulated anion channels (VRAC) (Kimelberg, 2005). Astrocytes can contribute to inflammation by releasing cytokines and chemokines (Downes and Crack, 2010) such as Interleukin-1 β (IL-1 β) which can contribute to increased stroke volume (Fogal et al., 2007). Both hypoxia and inflammation cause activation of the Nuclear Factor Kappa- β (NF- κ B) pathway which can suppress the expression of astrocytic glutamate uptake receptors (EAAT1 and EAAT2) (Rothstein et al., 1996) and impair the glutamate uptake by astrocytes. Therefore, astrocytes can contribute to potentiation of cell death in the penumbra through different mechanisms. On the other hand, astrocytes can also provide variety of supportive functions for recovery from ischemic injury by glutamate uptake, release of neuroprotective compounds such as adenosine (Parkinson and Xiong, 2004; Wardas, 2002), VEGF (Beck and Plate, 2009; Wick et al., 2002), matrix metalloproteinase-9 (MMP-9) (Thanabalasundaram et al., 2010), erythropoietin (Chavez et al., 2006), brain derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF-2) (Raff and Lillien, 1988) which can provide support during or after stroke recovery. In addition, astrocytes can detoxify ammonia by converting it to glutamine, as glutaminase synthase is mainly expressed by astrocytes in the brain (Norenberg and Martinez-Hernandez, 1979). Overall, astrocytes have variety of mechanisms that can be neuroprotective in ischemic brain injury.

TG2 can facilitate both cell survival and cell death by depending on the cell type and stressor (Gundemir and Johnson, 2009). This is the first study to indicate the possible role of TG2 in astrocytes in ischemic cell death. Our results suggest that TG2 ablation in mouse brain results in increased survival of astrocytes and astrocytic support of neurons. The decrease in stroke volume that we observed in $TG2^{-/-}$ mouse brain could be due to an increase in adaptive processes and/or suppression of pathological affects in the astrocytes.

TG2 is upregulated in response to ischemia (Filiano et al., 2010) and can activate the NF- κ B pathway (Lee et al., 2004). TG2 ablation via siRNA suppressed NF- κ B activity in HEK293 cells (Jang et al., 2009), and in TG2^{-/-} mouse embryonic fibroblasts (MEFs), hypoxia-induced NF- κ B activity was lower compared to the wild type mice (Kim et al., 2010). During hypoxia, NF- κ B suppresses the expression of glutamate transporters (EAAT-1 and EAAT-2) in astrocytes (Dallas et al., 2007) which can increase extracellular glutamate levels. Therefore, TG2 upregulation and further activation of NF- κ B pathway could lead to glutamate excitotoxicity due to suppression of glutamate transport. Therefore it can be speculated that in the TG2^{-/-} mouse brain there may be less NF- κ B activation after a stroke which facilitate cell survival. Additionally, NF- κ B activation is required for HIF-1 function in astrocytes (Rius et al., 2008) and can induce IL-1 β in astrocytes during hypoxia (Zhang et al., 2006). Given that decreased HIF-1 in astrocytes is protective against hypoxia (Vangeison et al., 2008) and IL-1 β can cause increased glutamate release from astrocytes in response to hypoxia which potentiates neuronal cell death (Fogal et al., 2007), it can be speculated that an attenuation of NF- κ B activation in astrocytes from TG2^{-/-} mice with a subsequent attenuation of downstream pathological events, may result in increased survival in ischemia. However, additional studies are required to explore these possible mechanisms.

Another mechanism that TG2 can contribute to ischemic cell death/survival is through matrix metalloproteinase-9 (MMP-9). TG2 overexpression reduces the MMP-9 protein expression and inhibits its activity (Ahn et al., 2008). Therefore, MMP-9 expression might be induced in TG2^{-/-} mouse brain. MMP-9 has both pro-survival and pro-cell death roles in ischemia. MMP-9 causes increased blood-brain barrier permeability and potentiates cell death during ischemia (Cunningham et al., 2005). On the other hand, MMP-9 cleaves and activates VEGF after ischemic injury which can potentiate cell survival (Zhao et al., 2006). Therefore, TG2 ablation might cause less penumbral cell death after stroke due to increased VEGF levels via increased MMP-9 activity. However, this might also cause increased blood-brain barrier permeability and lead to cell death during stroke. Therefore, this aspect of TG2 should be further studied to clarify whether TG2 exerts its pro-cell death or pro-survival affects through MMP-9.

Previously it has been suggested that increased transamidation activity of TG2 could contribute to potentiation of cell death. However, we don't know if the transamidation activity of TG2 is elevated in response to stroke in wild type mouse brain. Therefore, the contribution of transamidation activity in stroke damage needs to be examined in future studies.

In conclusion the results of these studies demonstrate that TG2 differentially modulates ischemic cell death in astrocytes and neurons. Further, astrocytes from TG2^{-/-} mice were not only more resistant to ischemic-induced cell death, but were also able to increase the survival of neurons. Within the last decade, it is becoming more evident that targeting astrocytes is a potential approach for developing a stroke therapy (Zhao and Rempel, 2010). Therefore the differential role of TG2 in astrocytic and neuronal cell survival/death in response to ischemia should be also considered when evaluating these possible stroke treatment strategies.

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Abbreviations

(TG2)	Transglutaminase 2
(MCAL)	middle cerebral artery ligation
(OGD)	oxygen-glucose deprivation
(TG2^{-/-})	TG2 knock out
(hTG2)	human TG2
(EPO)	erythropoietin
(VEGF)	vascular endothelial growth factor
(MMP-9)	matrix metalloproteinase-9
(NF-κB)	nuclear factor kappa B
(TTC)	triphenyltetrazolium chloride
(IL-1β)	interleukin 1β

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Highlights

- TG2^{-/-} mouse stroke volumes were significantly smaller.
- Primary neurons isolated from TG2^{-/-} mice were more vulnerable to ischemic insult.
- Primary astrocytes from TG2^{-/-} were more resistant to ischemic insult.
- Astrocytes from TG2^{-/-} protected neurons from ischemic cell death.

Figure 1. Relative transcript levels of other transglutaminases in $TG2^{-/-}$ mouse brain
mRNA transcript levels of TG1, TG2 and TG3 relative to mouse β -actin in both wild type and $TG2^{-/-}$ mouse brain. (N=6).

Figure 2. Macrovascular and microvascular structure analysis

A, Representative photos for wild type and $TG2^{-/-}$ mouse brain. Major arteries were stained with bromophenol blue for macrovasculature analysis. Circle of Willis is shown in the images. The angle between the red dotted lines was measured to determine localization of middle cerebral artery (MCA) relative to Circle of Willis. **B**, Angle of MCA determined from each mouse brain is shown as single data point on the graph. There is no significant difference between the two groups. ($N=5-10$) **C**, Immunohistochemistry of wild type and $TG2^{-/-}$ mouse cortex for microvasculature analysis. Brain sections were stained with FITC-lectin to label the blood vessels. Pictures were taken at 40 $\times$. **D**, The summation of total number of particles counted in each brain section was used to represent microvascular density. There is no significant difference between the two groups. ($N=3$).

Figure 3. Permanent middle cerebral artery ligation (MCAL)

MCAL was performed on wild type and TG2^{-/-} mice. Brains were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and sectioned into 1 mm thick slices. Infarct volume was calculated by using Image J program. TG2^{-/-} mice had significantly smaller infarcts compared to wild type mouse. (N=6 in each group).

Figure 4. Relative transcript levels of TG3 after stroke

A, B, mRNA transcript levels of TG3 relative to β -actin were determined 5h (**A**) and 24h (**B**) after MCAL on both ipsilateral and contralateral sides of wild type and TG2^{-/-} mouse brain. There was no difference between the groups. (N=4).

Figure 5. Fold transcript levels of enolase 1, VEGF and Noxa

A, B, Fold transcript levels of enolase 1, VEGF and Noxa were determined 5h (**A**) and 24h (**B**) after MCAL surgery on both ipsilateral and contralateral sides of wild type and $TG2^{-/-}$ mouse brains. Data is represented as fold induction of mRNA expression on the ipsilateral side relative to the contralateral side. There was no difference between the groups. (N=6 in each group).

Figure 6. OGD induced cell death in primary neuron, primary astrocyte cultures and neuron-astrocyte co-cultures

A, Resazurin cell viability assay was performed on wild type and TG2^{-/-} mouse primary neurons after OGD-reoxygenation treatment. Data is represented as percent cell viability relative to control samples that are kept under normoxic conditions. (N=7 in each group) **B**, Resazurin cell viability assay was performed on wild type and TG2^{-/-} mouse primary astrocytes after OGD-reoxygenation treatment. Data is represented as percent cell viability relative to control samples that are kept under normoxic conditions. (N=4 in each group) **C**, Resazurin cell viability assay was performed on wild type and TG2^{-/-} mouse primary neuron-astrocyte co-cultures after OGD-reoxygenation treatment. Data is represented as percent cell viability relative to control samples that are kept under normoxic conditions. (N=6 in each group).

Figure 7. OGD induced cell death in $TG2^{-/-}$ primary astrocytes transduced with TG2 expressing lentivirus

A, Representative immunoblots of TG2 expression in $TG2^{-/-}$ astrocytes transduced with empty control and TG2 expressor lentivirus (1st row). Forty micrograms of protein was loaded per lane. Cell lysates were blotted with actin as loading control (2nd row). **B**, Resazurin cell viability assay performed on $TG2^{-/-}$ astrocytes transduced with empty control and TG2 expressor lentivirus. Data is represented as percent cell viability relative to control samples that are kept under normoxic conditions. (N=4 in each group).