

Increased expression of tissue-type transglutaminase following middle cerebral artery occlusion in rats

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Abstract

Tissue-type transglutaminase (TG-2) has been implicated in neurodegenerative diseases. In this study, induction of TG-2 was studied in rats following transient middle cerebral artery occlusion. Alterations in 2,3,5-triphenylterazolium chloride staining revealed maximum infarction 3 days after injury. Measurement of mRNA transcript levels by real-time PCR analysis showed both forms of TG-2 mRNA peaking on day 5 after injury in ipsilateral cortex, with greater induction of the full-length TG-2 (TG-L) transcript than the truncated form of the TG-2 (TG-S) transcript. However, in the ipsilateral hippocampus, peak induction of both forms of TG-2 mRNA peaked 1 day after injury and to a lesser extent than observed in the ipsilateral cortex. Western blot analysis demonstrated that

TG-L protein expression progressively increased from 1 to 7 days after ischemia, with greater expression in cortex than hippocampus ($525 \pm 10\%$ vs. $196 \pm 8\%$ of control, respectively). However, expression of TG-S was not detected. These results demonstrate that increased TG-2 mRNA and protein expression occurs in a delayed fashion following ischemic injury. The temporal profile of TG-2 induction after ischemia was similar to that observed after traumatic brain injury (previously described), suggesting a similar role of TG-2 in both pathological conditions.

Keywords: brain injury, cross-linking, ischemia, transglutaminase.

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Stroke, the permanent or prolonged occlusion of an artery in the brain, is a major cause of death and the primary cause of adult disability in many countries. Arterial occlusion results in tissue infarction and neuronal death (Elkind and Sacco 1998). Thus, it is critical to develop therapeutic interventions to ameliorate this damage. However, the mechanisms underlying cell loss due to cerebral ischemia remain incompletely understood. Recent findings suggest that much of the neuronal death following stroke occurs as a result of apoptosis (Mattson 2000; Graham and Chen 2001). Cerebral ischemia leads to increased Ca^{2+} influx due to membrane depolarization (Kristian and Siesjö 1998), resulting in the activation of proteases (Buki *et al.* 2000; Beer *et al.* 2001) and tissue-type transglutaminase (TG-2) (Lai *et al.* 2001). These are pivotal events in apoptotic neuronal death (Facchiano *et al.* 2001; Hayes *et al.* 2001), in conjunction with mitochondrial dysfunction, release of cytochrome *c* and increased cytokine production (Bratton *et al.* 2000; Zipfel *et al.* 2000; Chu *et al.* 2002).

Tissue-type transglutaminase belongs to a family of Ca^{2+} -dependent transglutaminases (TGs) that catalyses the incorporation of a polyamine into a polypeptide-bound

glutamine, leading to the formation of an (γ -glutamyl) isopeptide bond (Greenberg *et al.* 1991). This isopeptide bond is resistant to proteolytic cleavage and may serve to not only stabilize proteins against degradation but also to alter their function. The most ubiquitously expressed member of the TG family is TG-2. It is abundantly expressed in the brain (Kim *et al.* 1999) and is induced in cultured cells by various agents including cytokines (Ikura *et al.* 1994) and cAMP (Maddox and Haddox 1988). The most potent inducers of

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Abbreviations used: AD, Alzheimer's disease; DEPC, diethylpyrocarbonate; MCAO, middle cerebral arterial occlusion; TG, transglutaminase; TG-2, tissue-type transglutaminase; TG-L, full-length tissue-type transglutaminase; TG-S, truncated form of tissue-type transglutaminase.

TG-2 are retinoids, which promote apoptosis in various cells including neurons (Ferrari *et al.* 1998; Lefebvre *et al.* 1999). Tissue-type transglutaminase has been implicated in numerous processes, including axonal regeneration, cellular differentiation, neurite outgrowth, apoptosis and neurodegeneration (Suedhoff *et al.* 1990; Eitan and Schwartz 1993; Piredda *et al.* 1999; Citron *et al.* 2001; Tucholski *et al.* 2001).

In addition to isopeptide bond formation, TG-2 has GTPase activity (Nakaoka *et al.* 1994). GTP binds at the COOH-terminal of full-length protein (Casadio *et al.* 1999) and inhibits its cross-linking activity. Recently, an alternatively spliced, short form (encoding ~70 kDa protein) of TG-2 induced by cytokine treatment was produced in cultured rat astrocytes and had greater activity at much lower Ca^{2+} concentrations (Monsonogo *et al.* 1997). The short form lacks a GTP-binding region and loss of GTPase activity correlates with increased cross-linking activity. This loss of function might have an impact on apoptotic neuronal death after brain injury. For the current study, we used a model of middle cerebral arterial occlusion (MCAO) to assess mRNA and protein levels of these two forms of TG-2 and examined how these changes correlated with the extent of ischemic injury.

Materials and methods

Middle cerebral artery occlusion

Transient occlusion of the right middle cerebral artery was achieved via intraluminal occlusion with a nylon suture (He *et al.* 2000). Adult male (250–300 g) Sprague-Dawley rats were anesthetized with an abdominal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Core body temperature was monitored continuously by a rectal thermistor probe and maintained at $37 \pm 1^\circ\text{C}$ by placing an adjustable heating pad beneath the rats. The right common carotid artery and right external carotid artery were exposed through a midline neck incision. A 3-0 monofilament nylon suture (25 mm long) was inserted through an arteriotomy of the right common carotid artery and gently advanced into the internal carotid artery to a point approximately 17 mm distal to the carotid bifurcation. Mild resistance to further advancement indicated that the suture had entered the anterior cerebral artery, thereby occluding the origin of the middle cerebral artery. After 1 h of occlusion, the nylon suture was withdrawn and the common carotid artery was ligated. The neck incision was closed with nylon suture. Sham-injured animals underwent an identical surgical procedure but did not experience occlusion. Appropriate pre- and post-injury management was maintained and these measures complied with all guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the Guide for the Care and Use of Laboratory Animals.

Histological assessment

Staining with 2,3,5-triphenylterazolium chloride was used to assess the extent of ischemic injury after MCAO. Following a 1-h occlusion, animals were killed immediately (0 days) or 1, 3, 5 or 7 days after injury with CO_2 and subsequently decapitated. Each

brain was removed and placed in a metallic brain matrix (Harvard Instruments, South Natick, MA, USA) for tissue slicing. A coronal section (2 mm thick) at the level of the optic chiasm was incubated for 30 min at room temperature in a solution of 2% 2,3,5-triphenylterazolium chloride in saline and then fixed in 10% formalin.

RNA purification

Ipsilateral (side of injury) cortices and hippocampi were rapidly dissected from animals at 1–7 days after occlusion and from sham-injured animals on day 5. At the appropriate time-points, animals were killed with CO_2 and subsequently decapitated. Total RNA was isolated using TRIzol reagent (Gibco BRL, Rockville, MD, USA). Isopropanol precipitation and ethanol washes were performed according to the manufacturer's instructions and samples were resuspended in 50–100 μL DEPC-treated water.

Reverse transcription

Total RNA (3 μg) was incubated with 1 μL oligo(dT) (0.5 mg/mL; GibcoBRL) at 70°C for 10 min and then at 4°C for 5 min. A RT reaction mixture was added to the RNA-oligo(dT) sample for a final volume of 20 μL , containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl_2 , 500 μM dNTPs, 10 mM dithiothreitol, 50 units SuperScript II reverse transcriptase (GibcoBRL) and 40 units RNaseOUT recombinant ribonuclease inhibitor (GibcoBRL). The sample was incubated at 42°C for 55 min, 70°C for 15 min for enzyme denaturation and then transferred to 4°C . Each sample was diluted to a final volume of 100 μL with DEPC-treated water.

Primer selection

Tissue-type transglutaminase-specific primers

All base pair designations refer to GeneBank locus AF106325, rat TG-2. 5'TG (ACTTTGACGTGTTGCCAC, bp 1470–1489) recognizes an upstream homologous sequence in full-length TG-2 (TG-L) and truncated TG-2 (TG-S) transcripts. 3'TG-L (CAAT-ATCAGTCGGGAACAGGTC, bp 1961–1982) recognizes a downstream TG-L mRNA-specific sequence. 3'TG-S (GCTGAGTCTGG, GTGAAGACACAG, bp 1861–1872 and 2083–2093) recognizes a downstream TG-S mRNA-specific sequence. 3'TG-S primer bridges the junction created by the absence of bp 1873–2082 (a sequence present exclusively in TG-L). The underlined 3' half of the 3'TG-S sequence will hybridize to both the TG-L- and TG-S-specific sequences while the full-length primer will hybridize only to the TG-S mRNA sequence.

GAPDH-specific primers

All base pair designations for GAPDH-specific primers refer to GeneBank locus AF106860. The upstream primer is designated 5'GPD (GGCTGCCTCTCTGTGAC, bp 903–921) and the downstream primer is designated 3'GPD (GGCCGCCTGCTTCAC-CAC, bp 1624–1641).

Semi-quantitative/light cycler PCR

Real-time PCR was performed using a Light Cycler rapid thermal cycler system (Roche Diagnostics, Indianapolis, IN, USA), as previously described (Tolentino *et al.* 2002). Reactions were performed in a 10- μL volume with 0.5 μM primers and 2.5 mM MgCl_2 . Other reagents, including nucleotides, Fast Start Taq DNA

polymerase and buffer, were used as provided in the Light-Cycler-FastStart DNA Master SYBR Green I reaction mix (Roche Diagnostics). The amplification protocol included a 5-min 95°C denaturation; one cycle with 95°C denaturation for 5 s, 65°C annealing for 10 s and 72°C extension for 35 s; one cycle with 95°C denaturation for 5 s, 62.5°C annealing for 10 s and 72°C extension for 35 s and then 30–40 cycles of 95°C denaturation for 5 s, 60°C annealing for 10 s and 72°C extension for 35 s. Detection of the fluorescent product occurred at the end of the 72°C extension periods. Specificity of the amplification product from each primer pair was confirmed by melting curve analysis of the PCR product and subsequent gel electrophoresis.

Quantification was performed by online monitoring for identification of the exact time-point at which the logarithmic linear phase could be distinguished from the background (crossing point). The crossing point is expressed as a cycle number.

Standard curve preparation and semiquantitative PCR analysis

The RT product from ipsilateral cortical RNA collected 3 days (when the initial mRNA induction was observed) after ischemic injury underwent serial dilution creating a standard curve of 100, 33, 11 and 3.7% of original real-time product. Each dilution from the standard curve was analyzed with the Light Cycler PCR using primer sets for TG-L, TG-S or GAPDH mRNA. For each primer set, a crossing point cycle number was determined for each dilution of the standard curve. Linear regression analysis of the logarithm of the dilution factor versus the crossing point cycle number generated a standard curve for each transcript-specific primer set. From the standard curve of each primer set, a crossing point cycle number could be converted to a relative amount of RNA. For individual samples, the crossing point cycle number was identified with the Light Cycler PCR. Using the standard curve for each primer set, the amount of TG-L, TG-S or GAPDH mRNA was determined. The amount of each transcript in sham animals was set at 100% and the level of expression in an occluded sample was calculated as a percent of sham expression.

Tissue lysis and protein purification

Cortical and hippocampal tissues were collected from animals 1–7 days after ischemic injury and from sham-injured animals 5 days after injury. At the appropriate time-points, animals were killed by CO₂ and subsequent decapitation. Ipsilateral and contralateral cortices and hippocampi were rapidly dissected and snap-frozen in liquid nitrogen. Tissue samples were stored at –80°C. Tissues were homogenized in a glass tube with a Teflon dounce pestle in 15 volumes of ice-cold triple detergent lysis buffer [20 mM Hepes, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1.0% IGEPAL 40 and 0.5% deoxycholic acid, pH 7.5] containing a broad-range protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). Samples were sonicated and centrifuged at 800 g for 5 min at 4°C. The supernatant fluid was then collected for western blot analysis.

Western blot analysis

Protein concentrations of tissue homogenates were determined by bicinchoninic acid microprotein assays (Pierce, Rockford, IL, USA) with albumin standards. Aliquots (20 µg) of each sample

were prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis by the addition of 2× loading buffer [1× loading buffer contains 125 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 4% sodium dodecyl sulfate, 0.01% bromophenol blue and 10% glycerol]. Samples with loading buffer were heated for 10 min at 100°C, centrifuged for 1 min at 10 000 g and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 7.5% Tris/glycine gels at 200 V for 1 h at 4°C. Following electrophoresis, fractionated proteins were transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA) in a transfer buffer containing 192 mM glycine, 25 mM Tris-HCl (pH 8.3) and 10% methanol at 100 V for 1 h at 4°C. Blots were blocked for 1 h at room temperature in 5% non-fat milk in TBST (20 mM Tris-HCl, 150 mM NaCl and 0.005% Tween-20, pH 7.5).

Immunoblots were probed with TG-100, a monoclonal anti-TG-2 antibody (LabVision, Fremont, CA, USA). Following overnight incubation at 4°C with the primary antibody (1 : 3000), blots were incubated for 1 h at room temperature in 3% non-fat milk/TBST containing a horseradish peroxidase-conjugated goat anti-mouse IgG (1 : 3000; Bio-Rad Laboratories, Hercules, CA, USA). Bound antibodies were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) on Kodak Biomax ML chemiluminescent film (Fisher Scientific International Inc., Hampton, NH, USA).

Statistical analyses

For the western blots, semiquantitative analysis was performed by computer-assisted densitometric scanning (Alpha Imager 2000 Digital Imaging System, Alpha Innotech Corporation, San Leandro, CA, USA). For each time-point studied, $n = 4$ per group. Data were acquired as integrated densitometric values and transformed to percentages of the densitometric levels obtained from sham-injured animals visualized on the same blot. Data were evaluated by two-tailed Student's *t*-test. All values are given as mean ± SEM. Differences were considered significant if $p < 0.05$.

Results

Histological assessment of ischemic injury

Coronal sections of rat brain (2 mm thick) were collected immediately to 7 days after transient MCAO and stained with 2,3,5-triphenylterazolium chloride (Fig. 1). Decreased 2,3,5-triphenylterazolium chloride staining was noted in areas of infarction. The maximal effect of occlusion was noted 3 days after ischemic injury, with a smaller infarct revealed at later time-points.

PCR analysis of tissue-type transglutaminase transcripts

A previous report described the use of a semiquantitative PCR analysis to measure the expression of TG-L and TG-S mRNA in rat cortex and hippocampus after traumatic brain injury (Tolentino *et al.* 2002). The same technique was used to measure the expression of these transcripts in rat cortex and hippocampus after ischemic injury. Figure 2 shows the time-course of TG-L and TG-S mRNA expression in



Fig. 1 2,3,5 -Triphenylterazolium chloride staining of rat brain coronal sections after ischemic injury. 1–7 days after transient right middle cerebral arterial occlusion, rat brains were dissected and sectioned in the coronal plane. Areas of infarcted tissue were revealed by decreased staining. Maximal effect of occlusion was noted 3 days after injury with a smaller infarct revealed at later time-points.

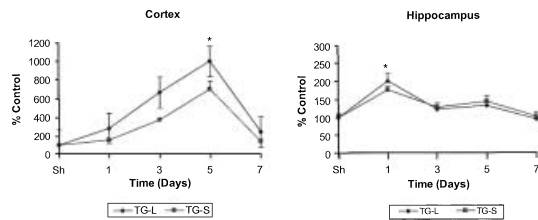


Fig. 2 Semi-quantitative PCR analysis of TG-L and TG-S mRNA expression in ipsilateral cortex (a) and hippocampus (b). TG-L (◆) and TG-S (■) mRNA levels were calculated as a percent of sham (Sh) control. In cortex, maximal TG-L and TG-S mRNA expression was noted 5 days after ischemia while, in hippocampus, maximal expression was noted 1 day after ischemia ($*p < 0.05$).

ipsilateral cortex and hippocampus after ischemia. In ipsilateral cortex, maximal and significantly elevated expression of TG-L and TG-S mRNA was observed 5 days after injury ($990 \pm 130\%$ and $690 \pm 90\%$ of control, respectively). In the hippocampus, maximal and significantly elevated TG-L and TG-S mRNA levels were observed 1 day after injury ($200 \pm 20\%$ and $175 \pm 11\%$ of control, respectively).

Western blot analysis of cortical tissue-type transglutaminase expression after ischemic injury

Total protein was prepared from ipsilateral and contralateral rat cortex after sham and ischemic injury. Figure 3(a) shows TG-2 protein expression in rat cortex 1–7 days after ischemic injury. Using the TG-100 (monoclonal anti-TG-2) antibody, a distinct TG-2 protein band at ~ 79 kDa was identified, corresponding to TG-L. However, TG-S (~ 70 kDa) was not identified using this antibody. The expression of TG-L was increased in a time-dependent manner compared with the sham-injured control. Western blot data were quantified by densitometric analysis (Fig. 3b). Levels of TG-L expression were calculated as a percent of the sham control. The induction of TG-L was observed 3–7 days after cortical occlusion with maximum induction on day 7 ($525 \pm 10\%$ of control). Comparing expression between matched ipsilateral

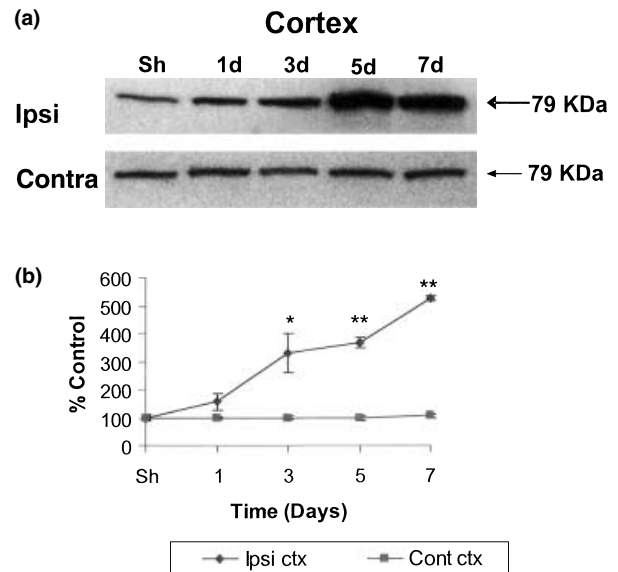


Fig. 3 (a) A representative western blot showing TG-2 expression in cortical protein samples using the anti-TG-2 antibody (TG-100). Samples were collected from injured animals 1, 3, 5 and 7 days after ischemic injury and from sham-injured (Sh) animals. TG-L (79 kDa) expression was increased in a time-dependent manner, while TG-S (70 kDa), was not identified in these samples, even at longer exposures. The TG-L protein expression in contralateral (Contra) samples remained the same. (b) Data from multiple ($n = 3$) western blots were acquired as densitometric values and transformed to percentages of the densitometric values obtained from control samples (sham-injured animals). Quantitative analysis of western blots showed increases in protein level following injury in a time-dependent manner on days 3, 5 and 7 compared with control animals (Sh). Comparing TG-L expression from ipsilateral (Ipsi) and contralateral cortex at each time-point, increased levels of expression were statistically significant at 3–7 days in the ipsilateral cortex ($*p < 0.05$, $**p < 0.005$).

and contralateral cortical tissues, statistically significant elevated expression was observed at 3, 5 and 7 days. Contralateral samples that showed no change in TG-2 expression were used as equal loading controls.

Western blot analysis of hippocampal tissue-type transglutaminase expression after ischemic injury

Total protein was prepared from ipsilateral and contralateral rat hippocampus after ischemic and sham injury and the expression of TG-2 was examined using TG-100. Figure 4(a) shows TG-2 protein expression in rat hippocampus 1–7 days after ischemic injury. Similar to western blot analyses of cortical samples, only TG-L (~79 kDa) was identified. The expression of TG-L protein was increased in a time-dependent manner from days 3 to 7 compared with the sham control. However, the induction level was lower than in ipsilateral cortex. Quantitative densitometric analysis (Fig. 4b) revealed maximum expression at 7 days after injury ($196 \pm 8\%$). Comparing expression between matched ipsilateral and contralateral hippocampal tissues, statistically significant elevated expression was observed at 3, 5 and 7 days after injury. Contralateral samples that showed no change in TG-2 expression were used as equal loading controls.

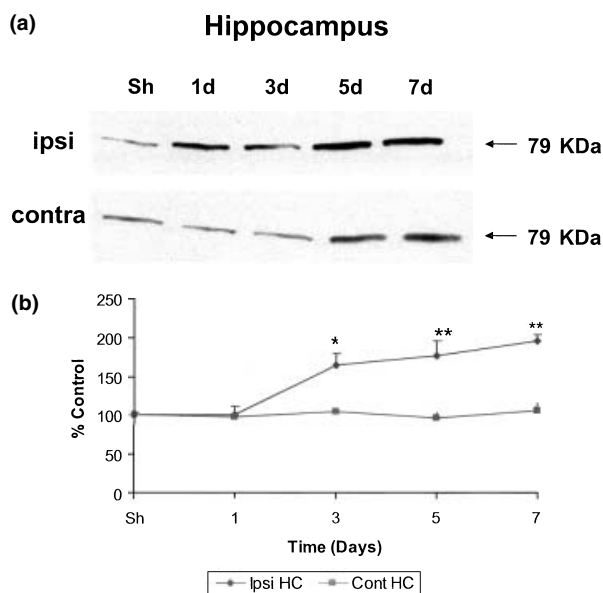


Fig. 4 (a) A representative western blot showing TG-2 expression in hippocampal protein samples using the anti-TG-2 antibody (TG-100). Samples were collected from 1, 3, 5 and 7 days after ischemic injury and sham-injured animals. TG-L (79 kDa) protein expression increased in a time-dependent manner compared to sham-injured controls, while TG-S (70 kDa) was not identified, even with longer exposure time. (b) Data from multiple ($n = 3$) western blots were acquired as densitometric values and transformed to percentages of the densitometric values obtained from control samples (sham-injured animals). Quantitative analysis of western blots showed increases in protein level following injury in a time-dependent manner on days 3, 5 and 7 compared with control animals (Sh). Comparing TG-L expression from ipsilateral (Ipsi HC) and contralateral (Cont HC) at each time-point, increased levels of expression were statistically significant at 3–7 days in ipsilateral hippocampus (* $p < 0.05$, ** $p < 0.005$).

Discussion

The TG family has been studied in a variety of injury models. The current study provides the first evidence of TG-2 induction in response to cerebral ischemia in a trend similar to that observed after traumatic brain injury (Tolentino *et al.* 2002), suggesting that TG-2 may be a good predictor for the pathophysiology accompanying traumatic and ischemic brain injury. Moreover, in a model of spinal cord ischemia, TG activity underwent a transient increase that declined to control levels after 1 week (Fujita *et al.* 1995). The TG-2 protein and transcript expression were observed after spinal cord injury and both the short- and long-form transcripts were identified after injury (Festoff *et al.* 2002). In the superior cervical ganglion, TG activity was increased within 1 h of axotomy and returned to baseline after 24 h (Gilad *et al.* 1985; Ando *et al.* 1993). The TG activity also increased in the vagus nerve after crush injury (Tetzlaff *et al.* 1988).

The role of TG-2 induction in the neural response to injury is not well understood; however, based on other experimental injury models, both damaging and protective functions can be proposed. For example, exogenous application of TG to injured optic nerve promoted axonal regeneration and recovery of visual-evoked responses (Eitan *et al.* 1994). In SH-SY5Y neuroblastoma cells, TG-2 stimulated neuronal differentiation (Tucholski *et al.* 2001). It was proposed that the supportive effects of TG treatment were due to stabilization of matrix proteins or protection of growing axons from oligodendrocyte-mediated growth inhibition. It had been previously shown that TG dimerizes interleukin-2 and that interleukin-2 dimers were cytotoxic to cultured oligodendrocytes (Eitan and Schwartz 1993).

Several studies have demonstrated the dual role of TG-2 in neuronal life and death processes (Im *et al.* 1997; Gill *et al.* 1998; Casadio *et al.* 1999). Increase in the TG-S transcript predicts production of a more active enzyme. As GTP is inhibitory to the cross-linking activity of TG-2, loss of this region presumably results in a switch of TG-2 function to programmed cell death with increases in cross-linking and production of aggregated proteins (Norlund *et al.* 1999; Citron *et al.* 2001, 2002). Our studies have demonstrated an increased message level of TG-S after MCAO; however, its protein expression was not detected, suggesting that TG-S protein may be expressed at an undetectable level. However, TG-S protein expression was recently detected in the spinal cord injury model by using a different set of antibodies (Festoff *et al.* 2002). Apoptotic neuronal death has been widely documented after ischemic brain injury (Mattson 2000; Graham and Chen 2001) and induction of TG-S mRNA expression may increase apoptotic neuronal death and cross-linking activity. Further understanding of the regulation of TG-2 transcription may provide additional opportunities to control TG-2 expression as a potential therapy against

inappropriate apoptosis and cross-linking activity that may lead to neurodegenerative diseases. In addition, the cell type localization and pathophysiology of TG-2 induction bear further examination. Tissue-type transglutaminase may be important not only for subsequent cell death after brain injury but also for compensatory mechanisms of other brain systems as they respond to the injury.

Tissue-type transglutaminase expression has been implicated in the cellular pathogenesis of Alzheimer's disease (AD) (Lesort *et al.* 2000; Citron *et al.* 2001) and increased TG-2 mRNA, protein expression and TGase activity have been observed in brains of AD and Parkinson's disease patients compared with controls (Citron *et al.* 2001, 2002). The TG-S isoform message was absent in normal brains but present in AD patients and accompanied by an increase in cross-linking of disease-specific proteins, such as tau and α -synuclein (Citron *et al.* 2002). If TG activity is physiologically relevant to the development of AD, then TG induction after MCAO may contribute to the epidemiological observation that a subset of individuals who experience ischemia are at a greater risk of developing AD. Recent advances suggest that cerebral ischemia may promote AD (Kalaria 2000). The underlying pathophysiological mechanisms of AD and stroke are similar (Blass and Ratan 2003). Cerebral ischemia may promote AD by triggering the development of senile plaques and neurofibrillary tangles (Vermeer *et al.* 2003). Further, MCAO increases the expression of presenilin, amyloid precursor protein (APP) and Apolipoprotein E (APE) (Pennypacker *et al.* 1999; Pluta 2000). These studies suggest that cerebral ischemia may cause cellular and molecular events that have neurodegenerative consequences and subsequently lead to the development of AD (Pennypacker *et al.* 1999; Kalaria 2000; Pluta 2000; Blass and Ratan 2003; Vermeer *et al.* 2003). The induction of both AD-related proteins and TG-2 following brain injury provides a potential mechanism by which cerebral ischemia may contribute to AD pathology. This hypothesis awaits further *in vivo* and *in vitro* experiment.

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