Differential Expression of Multiple Transglutaminases in Human Brain

INCREASED EXPRESSION AND CROSS-LINKING BY TRANSGLUTAMINASES 1 AND 2 IN ALZHEIMER’S DISEASE*  

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The transglutaminase (TGase) family of enzymes, of which seven different members are known in the human genome, participate in many biological processes involving cross-linking proteins into large macromolecular assemblies. The TGase 2 enzyme is known to be present in neuronal tissues and may play a role in neuronal degenerative diseases such as Alzheimer’s disease (AD) by aberrantly cross-linking proteins. In this paper, we demonstrate by reverse transcriptase-polymerase chain reaction and immunological methods with specific antibodies that in fact three members, the TGase 1, TGase 2, and TGase 3 enzymes, and are differentially expressed in various regions of normal human brain tissues. Interestingly, the TGase 1 and 3 enzymes and their proteolytically processed forms are involved in terminal differentiation programs of epithelial cell development and barrier function. In addition, we found that the levels of expression and activity of the TGase 1 and 2 enzymes were both increased in the cortex and cerebellum of AD patients. Furthermore, whereas normal brain tissues contain ~1 residue of cross-link/10,000 residues, AD patient cortex and cerebellum tissues contain 30–50 residues of cross-link/10,000 residues. Together, these findings suggest that multiple TGase enzymes are involved in normal neuronal structure and function, but their elevated expression and cross-linking activity may also contribute to neuronal degenerative disease.

Transglutaminases (TGases)* (EC 2.3.2.13) are calcium-dependent cross-linking enzymes (1–4) which are responsible for blood clotting (5), apoptosis (6, 7), seminal vesicle coagulation (8), cataract formation (9), extracellular matrix and bone formation (10, 11), and cornified envelope formation and barrier function in stratified squamous epithelia (12–16). Currently, seven different TGases are known (4), including TGase 1 (106 kDa, mostly membrane-bound, widely expressed in epithelia), TGase 2 (80 kDa, soluble, ubiquitously expressed), TGase 3 (77 kDa, soluble, expressed mostly in epithelia), TGase 4 (80 kDa, soluble, expressed mostly in prostate), band 4.2 (an inactive structural protein expressed in some cells), factor XIIIa (80 kDa, soluble, circulating blood cells), and “TGase X” expressed in epithelial tissues.

A number of reports have described the presence of TGase activity in regional and subcellular tissue locations and the presence of TGase substrates in the nervous system (17–21). In the peripheral nervous system, TGase activity was reported in sympathetic postganglionic motor neurons (18) and activity was increased in sheath cells in the distal portion of peripheral nerves following nerve injury (22). However, the nature of the TGase enzyme was not characterized. In the adult human central nervous system, only the TGase 2 enzyme has been identified by immunohistochemical studies (19). In neurons of both the peripheral and central nervous systems, several different bands of TGase were observed (21). Interestingly, in one set of experiments, about half of the TGase activity of mammalian brain homogenates was measured in the soluble (cytosolic) fraction, and the remainder was presumably associated with the membrane fraction (17). Furthermore, TGase activities with different biochemical characteristics were reported in sympathetic postganglionic motorneurons of the rat peripheral nervous system (23) and in the adult human central nervous system (24) although the nature of the TGases was not identified.

One possible explanation for these various findings is that there may be multiple different TGases in the neuronal system. In this study, examination of TGase expression in human brain tissue has revealed expression of not only TGase 2 but also of TGases 1 and 3 which are commonly found in stratified squamous epithelia (24). The functional role of TGases in the normal brain is not yet clear, but reports have documented that TGases may be involved in senile plaque formation in Alzheimer’s disease (AD)/24, 26–29. We extend an earlier report (29) to show that the levels of both TGases 1 and 2 are changed and increased in various parts of the brain in AD disease concomitant with significant levels of isopeptide cross-link formed by these enzymes.

MATERIALS AND METHODS

Tissue Preparation—Normal human brain tissue segments (autopsy samples of healthy male subjects dead by accidents) from cerebral cortex, amygdala, and corpus callosum were obtained from Chungnam National University Hospital, Korea. For comparative studies of TGase expression between normal and AD brain tissues, several tissue specimens of frontal lobe and cerebellum of normal age matched (80–81 years old, post-mortem time 10.5–12.7 h) individuals, and of AD patients (64–71 years old, post-mortem time 9.4–11.7 h), were obtained from Dr. Daniel Brady, National Institute of Aging.

The frozen brain specimens were homogenized using a Teflon pestle.
with 0.1 N Tris acetate (pH 7.5), 1 mM EDTA, containing protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 50 μg/ml calpain inhibitor I, 100 μg/ml bestatin, and 1 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was used for the soluble (cytosolic) fraction. The pellet remaining after centrifugation was extracted with the same buffer as above containing 1% Triton X-100 for 10 min at room temperature, and pelleted again. This procedure released the membrane-bound TGase activity into the supernatant. No further TGase activity could be detected following a second treatment and centrifugation. Both the cytosolic and membrane-bound fractions were used for TGase activity assays and immunoblotting assays with TGase 1, 2, and 3 antibodies. To prepare total RNA for RT-PCR, control and AD brain tissues were homogenized directly in a glass tube using a Polytron homogenizer by adding TRIZOL reagent (100 mg/ml) (Life Technologies, Inc., Gaithersburg, MD) using the manufacturer’s instructions.

**Conditions for TGase Assay—** A modified TGase assay method was used to determine the enzymic activity by measurement of the incorporation of [1,4-3H]putrescine into succinylated casein 1). The samples were mixed in a reaction mixture (0.5 ml) containing 0.1 N Tris acetate (pH 7.5), 1% succinylated casein, 1 mM EDTA, 0.5% Lubrol PX, 5 mM dithiothreitol, 0.15 mM NaCl, and 0.5 μCi of [3H]putrescine (NEN Life Science Products Inc., Wilmington, DE) (118 Ci/mmol). Following incubation at 37 °C for 1 h, the reaction was terminated by adding 4.5 ml of cold (4 °C) 7.5% trichloroacetic acid. The trichloroacetic acid-insoluble precipitates were collected onto GF/A glass fiber filters, washed with cold 5% trichloroacetic acid, dried, and counted.

**RT-PCR Reaction of TGases with Normal Human Brain mRNA—** The RT-PCR primers were designed in the 3’-noncoding regions of very low homology between human TGase family members. The specificity of priming was confirmed with human foreskin mRNA (data not shown). The PCR primer sequences are: TGase 1 sense strand (5’-GATTGCTCTCAAGAACCCCCCTCCC-3’), TGase 1 antisense strand (5’-CTCATGCTAGCTCCAGCTCTAACCAGC-3’); TGase 2 sense strand (5’-CTGTTGAGAATTCACCACCAGCTTGT-3’); TGase 2 antisense strand (5’-TCTCGAAGTTCACCACCAGCTTGT-3’); TGase 3 sense strand (5’-AGCCAGTTATCAACAGCTAC-3’); TGase 3 antisense strand (5’-GACTCCAGTCCCATTGCTC-3’); TGase X sense strand (5’-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3’); TGase X antisense strand (5’-TGATTGCAGGGAACTTGTTGCAGG-3’); TGase 1 antisense strand (5’-GATTGTCTGATTGTCTGATTGTCT-3’); factor XIIIa antisense strand (5’-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3’); and β-actin sense strand (5’-GAACCTGTGAACGTGCAGATGCTCTTC-3’), β-actin antisense strand (5’-TTTATGAGATTGTATGATGTTG-3’); factor XIIIa antisense strand (5’-CATGCTGCTCAGCTACATTAGGAC-3’), factor XIIIa sense strand (5’-GATTGACTGCTCTACAGCCTCAGTCCATG-3’), TGase X sense strand (5’-ATGGCCCAAGGCTGACTAGGTCGGCC-3’), TGase X antisense strand (5’-GCTCAGCCTCAGTTGATGTGAC-3’); and β-actin sense strand (5’-ATCCTGGCCACACACACCTGCTACATGAGCCTG-3’), β-actin antisense strand (5’-GCTCAGTACTCCTGCTTGCTGATCACCATGCTC-3’). The reverse transcription reaction of the mRNA was performed at 42 °C with avian myeloblastosis virus reverse transcriptase (CLONTECH, Palo Alto, CA). The PCR reaction was done with conditions of one cycle of 95 °C (2 min), 30, 35, or 37 cycles each of 95 °C (30 s), 55 °C (30 s), 72 °C (30 s), and finally one cycle of 72 °C (7 min). The PCR products were resolved on 4% agarose gel and stained with ethidium bromide. Product size is shown on the right as base pairs. These data are representative of tissues from eight normal individuals.

**Material and Methods—** The three specific affinity purified TGase antibodies used were: 1) polyclonal anti-human TGase 1 made in goats (dilution 1:200); 2) polyclonal anti-human TGase 2 made in rabbits (dilution 1:400); and 3) polyclonal anti-human TGase 3 made in rabbits (dilution 1:200). In addition, specific polyclonal antibodies were elicited against bacterially expressed forms of the C-terminal regions of human TGase 2 (residues 523–687) and human TGase 3 (residues 470–692). The specificities were confirmed by immunoprecipitation and Western blotting (data not shown). Human TGase 1, 2, and 3 proteins expressed in bacteria (35, 36, 38) were used to block the action of the TGase antibodies as negative controls.

**Immunoslot Blotting—** To obtain ranges suitable for accurate quantitation by the PhosphorImager, the cytosolic and membrane-bound fractions of neuronal tissue extracts were serially diluted in the range of 25 μg to 1.5 μg, and were applied into slot chambers followed by twice washing with Tris-buffered saline. Western blotting was performed as established previously (37). The concentrations of antibodies were 5 μg/ml for primary antibodies and 0.1 μg/ml for secondary antibodies. The blots were then developed by an enhanced chemiluminescence method (Pierce), and exposed for quantitation.

**Isolation and Quantitation of Isopeptide Cross-link—** Several frozen tissue sections (20 μm thickness) were extracted in a solution containing 2% (w/v) SDS and 1% dithiothreitol and boiled vigorously for 10 min. Insoluble proteins were sedimented at 13,500 × g for 5 min. These pellets were extracted three more times (38, 39). Samples were then subjected to total enzymic hydrolysis to release the intact Nε-(γ-glutamyl)lysine isopeptide cross-link, which was resolved and quantitated by amino acid analysis (38). As the amount in normal brain tissues was very low, in some experiments, we digested the entire tissue section instead.

**RESULTS**

We have examined the distribution of mRNA and expressed protein of the several known TGase gene products in four different tissue regions of human brain: amygdala, cerebellum, corpus callosum, and cortex.

**Fig. 1. Differential expression of TGase 1, 2, and 3 mRNAs in normal human brain.** RT-PCR of TGase 1, 2, and 3 showed different expression levels in the three different parts of brain. TGase 1 was abundant in corpus callosum, TGase 2 was ubiquitous, and TGase 3 was expressed highly in amygdala. The results of RT-PCR showed the amount of product per 0.1 μg of mRNA after 35 cycles except β-actin, 25 cycles. The products were run on 2% agarose gel and stained with ethidium bromide. Product size is shown on the right as base pairs. These data are representative of tissues from eight normal individuals.

**Multiple Transglutaminases in Human Brain**

[Image 360x556 to 503x729]
scripts (Fig. 1): TGase 4, band 4.2, factor XIIIa, and TGase X mRNAs were not detected (data not shown). Interestingly, the amount of the three TGase transcripts varied in the different parts of the brain: the expression of TGase 1 was five times higher in the corpus callosum and cerebellum (data not shown) than in the other parts tested; the expression of TGase 3 was more than 10 times higher in the amygdala; but expression of TGase 2 was essentially the same in all tissues tested (Fig. 1). We were unable to confirm an earlier report of factor XIIIa expression in brain tissues (2).

**Immunohistochemical Localization of TGase 1, 2, and 3 Enzymes in Normal Adult Human Brain**—We performed immunohistochemical staining of the four different parts of normal adult human brain including amygdala (Fig. 2, A–C), cerebral cortex (Fig. 2, D–F and J–L), corpus callosum (Fig. 2, G–I), and cerebellum (data not shown) with specific antibodies for the TGase 1 (Fig. 2, A, D, and G), 2 (Fig. 2, B, E, and H), and 3 (Fig. 2, C, F, and I) enzymes. Staining of the tissue sections with preimmune sera showed no cross-reactivity (Fig. 2L for TGase 1). In the amygdala, TGase 3 was highly expressed in the cytoplasm of pyramidal cells (Fig. 2C), whereas a much weaker diffuse staining was seen in the pyramidal cells by TGases 1 (Fig. 2A) and 2 (Fig. 2B). In the cerebral cortex and cerebellum (data not shown), TGases 1 and 2 stained the cytoplasm and dendritic processes of pyramidal cells in the gray matter (Fig. 2, D and E), but TGase 3 was not detectable (Fig. 2F). In the corpus callosum, staining for TGase 1 (Fig. 2G) was notably stronger than for TGases 2 and 3 (Fig. 2, H and I), although the staining of TGases 2 and 3 was somewhat stronger in the corpus callosum than the cerebral cortex (Fig. 2, H and I). In controls in cortex tissue, we used a specific antibody for the intermediate filament glial fibrillary acidic protein, which stained only glial cells (Fig. 2J), and an antibody for the intermediate filament neurofilament heavy chain, which stained only axons (Fig. 2K). Although these data indicate that TGase expression in neuronal cells is restricted to the cell bodies, we cannot rigorously exclude the possibility of expression and localization in the axons. Similarly, our data show that TGase expression is very low or undetectable in glial cells and astrocytes, although one earlier report has described the presence of TGase 2 in cultured rat astrocytes (40). Together, these observations correlate well with the RT-PCR data of Fig. 1.
1, and afford robust evidence for the expression of these three members of the TGase enzyme family in human brain tissues.

Elevated Expression of Especially TGase 1 in AD Brain Tissues—Next, we studied TGase expression in cerebellum and cerebral cortex tissues from normal and AD patients. Semi-quantitative RT-PCR was used to estimate the amounts of mRNAs for these TGases: in order to ensure a linear relationship between the amount of PCR product and amount of total RNA, we used 20 cycles for β-actin and TGase 2, 27 cycles for TGase 1, and 35 cycles for TGase 3. The amounts were standardized with respect to the amount of β-actin mRNA. In both normal tissues, the amount of TGase 2 mRNA was severalfold higher than for TGase 1, but the amount for TGase 3 was too low to be statistically significant (Fig. 3). Notably, there was a 70% increase in the amount of TGase 1 mRNA in the cerebellum of six AD patients tested, but it was unchanged in the cortex of eight patients, including the same six. Likewise, there was a 65–75% increase in the amount of TGase 2 mRNA in both tissues of AD patients. These results for TGase 2 are generally similar to those of Johnson (29).

We then examined the subcellular localization of the TGase activity, since the foregoing data lack the resolution to determine whether the TGases are cytosolic or membrane-bound. Fig. 4 shows that about 20% of the total enzymic activity in control cortex and cerebellum resided in the membrane fraction, and was increased about 2-fold in the cerebellum tissue of the AD patients. The majority of the TGase activity resided in the cytosolic fraction, and was increased about 3-fold in the cerebellum of AD patients. Both sets of data are consistent with Fig. 3.

To confirm the identity of these activities, we performed Western slot blotting experiments using specific anti-TGase 1 and 2 antibodies in cortex (Fig. 5A) and cerebellum (Fig. 5B) tissues from post-mortem time-matched normal and AD individuals. Total data from three matched pairs (cortex) or five pairs (cerebellum) were quantitated by scanning densitometry and are summarized in Table I. (Comparable data for the TGase 3 enzyme were not possible because the expression levels were too low.) First, we were unable to detect measurable amounts of TGase 2 antigen in the membrane fractions in either tissue. Second, in the cortex, TGase 1 levels of both the membrane and cytosolic fractions were increased manyfold in AD in comparison to normal controls, whereas TGase 2 levels were increased only about 2-fold in the cytosolic fraction. Third, while there were only modest increases of TGase 1 and 2 levels in the cytosolic fraction of the cerebellum of AD tissues, there was a 10-fold increase of TGase 1 in the membrane fraction. We
cannot rigorously exclude the possibility that these differences, especially the TGase 1 levels in AD tissue, are due to delays in postmortem sample processing, but this seems unlikely since the samples were carefully matched.

Immunohistochemical staining of cerebellum tissue using anti-TGase 1 (Fig. 6, A and B) and anti-TGase 2 (Fig. 6, C and D) antibodies consistently showed that TGase 1 and 2 were more strongly positive in cerebellum and cortex (not shown) tissues of AD patients (Fig. 6, B and D) than in the normal controls (Fig. 6, A and C). Interestingly, TGase 1 showed cell peripheral and cytoplasmic staining while TGase 2 showed generalized cytoplasmic staining. In addition, TGase 1 staining in AD tissue showed unusual protein tangles (UT), and TGase 2 showed unusual protein fibrils (UF). Similar data were obtained in several other tissue samples.

Isolation of Isopeptide Cross-link from High Molecular Weight Proteins Recovered from AD Tissues—Finally, we wanted to know whether the increased expression of TGases and TGase activities contributed to the formation of cross-linked protein material. Isopeptide cross-link amounts were measured in two ways. First, total enzymic digestion of intact tissue sections revealed ~1 residue of cross-link/10,000 residues in normal cerebellum or cortex tissue sections, but much larger amounts in tissue sections from AD patients. To quantitate this more accurately, tissue sections were exhaustively boiled in buffer containing SDS and reducing reagents. Whereas we were able to isolate only traces of insoluble proteins from cerebellum and cortex tissues of normal individuals (yields <1 μg/mg of total protein), we recovered 35 ± 15 and 75 ± 20 μg/mg of insoluble proteins from these two tissues of AD specimens, respectively. Following total enzymic hydrolysis these insoluble proteins contained 2 residues of cross-link/10,000 residues in normal tissues, but 30 and 50 residues/10,000, respectively, in the two AD tissues. By way of analogy, we have found 80–100 residues/10,000 residues in skin specimens (38, 39).

**Discussion**

**Multiplicity of TGase Activity in the Nervous System**—The purpose of the present experiments has been to identify the enzyme(s) which are responsible for the TGase activity in brain. We have provided robust evidence for the presence of multiple functional TGases in brain tissues. Of these, the TGase 2 enzyme was the most abundant (Fig. 5), as expected from several earlier studies, but significant amounts of the TGase 1 and lesser amounts of the TGase 3 enzyme are also present. Furthermore, we have provided evidence of differential expression of these TGase enzymes in different regions of the brain (Figs. 1–5).

**The TGase 1 and 3 Enzymes**—These two enzymes are abundantly expressed in terminally differentiating stratified squamous epithelia in response to elevated Ca\(^{2+}\) levels, and play important roles in barrier function by cross-linking structural proteins. Major aspects of their biochemical properties and substrate proclivities are now well understood (12–16). The ~77-kDa TGase 3 enzyme is typically located in the cytosol but is proteolytically processed during Ca\(^{2+}\) -induced terminal differentiation into a functional 50/27-kDa complex (33, 41). Most of the TGase 1 in epithelial cells exists as a ~100-kDa membrane-bound protein of very low specific activity, but during terminal differentiation, some may be activated by proteolytic processing into a 67/33/10-kDa form of very high specific activity, and some intact low specific activity or higher specific activity processed forms are released into the cytosol (37, 42). Thus cytosolic or membrane fractions of epithelial cells may hold large amounts of TGase 1 protein of either low or high activity depending on the degree of proteolytic activation. In contrast, however, the TGase 2 enzyme is destroyed by proteolysis (reviewed in Refs. 1–4). Thus proteolytic activation of TGase 1 can cause major changes in total TGase activity in

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**Table I**

| Frontal cortex | C | AD | C | AD | C | AD | C | AD | C | AD |
|---------------|---|----|---|----|---|----|---|----|---|----|
| TGase 1 Cytosol | 4 ± 3 | 31 ± 3 | 2 ± 1 | 26 ± 2 | 41 ± 3 | 83 ± 6 | 0 | 0 |
| TGase 1 Membrane | 21 ± 2 | 30 ± 2 | 3 ± 1 | 39 ± 2 | 30 ± 2 | 43 ± 3 | 0 | 0 |
| TGase 2 Cytosol | 20 | 24 | 1 | 6 |
| TGase 2 Membrane | 32 | 60 | 0 | 2 |

**Fig. 6. Immunohistochemical study of TGase 1 and 2 in AD cerebellum tissues.** The sections were from normal control (A and C) and AD (B, D, and E). The antibodies used are: anti-TGase 1 (A and B), anti-TGase 2 (C and D), and negative control (E). Both anti-TGase 1 and 2 stained neurons, and showed stronger positive reaction in AD in comparison to normal. The anti-TGase 1 staining showed cell peripheral staining (A and B), whereas anti-TGase 2 staining showed generalized cytoplasmic staining. Note in B the presence of unusual tangles (UT), and unusual fibrils (UF) in D (arrowheads). Nuclei are stained blue-green. The data shown are representative of three normal individuals and four AD patients. Scale bars, 50 μm.
cells. With this background, it is to be expected that substantial increases in total TGase activity could occur in normal or abnormal neuronal cells as a result of proteolytic activation of TGases 1 and/or 3.

**Complexity of Brain TGases**—Previous reports have described the complexity of brain TGases in terms of molecular size (67–100 kDa) (21, 43) and biochemical properties (17, 24). Furthermore, there is evidence that TGase activities reside in cytosolic and/or membrane compartments (22), and that membrane-associated brain TGases contain latent activity which can be activated with thiol reagents, thrombin, proteases, and physical agents (high salt, glycerol etc.) (21, 43). In another study, a significant degree of cross-linking of endogenous protein substrates occurred in synaptic membranes (44). There is evidence from studies of isolated superior cervical and nodose ganglia that intracellular TGase activities can be induced and rapidly activated by treatments including varatridine, high extracellular potassium (45), and acetylcholine (46). These agents result directly in an influx of Ca^{2+}. Such induction and rapid activation phenomena may occur by both protein synthesis-dependent and -independent mechanisms. Thus while unambiguous evidence has existed for some time on the presence of the TGase 2 enzyme (17, 21, 28, 29), it is somewhat difficult to reconcile these reported complexities with the known properties of the TGase 2 enzyme. Our new data reveal that there is significant TGase 1 and 3 activity in brain tissues. Indeed, many of the known properties of the cytosolic and membrane-associated forms of the TGase 1 and 3 enzymes and their activation in vivo concord well with these various reports on the complexity of TGases in brain tissues.

A recent report has documented the presence of a novel cytosolic TGase 2-like enzyme in cultured rat astrocytes (40) but we could not identify its mRNA in adult human brain tissues (data not shown). Furthermore, in contrast to our present work, previous studies have documented the presence of the factor XIIIa enzyme in brain tissues (2), but we think this may be due to contamination from residual blood in the specimens tested.

**Distribution of TGases 1, 2, and 3 in Normal Adult Human Brain**—We found that the mRNA and protein for TGase 2 were abundantly expressed in each of the four tissue types studied here. This is to be expected from many published reports and since it is an essential housekeeping protein involved both in GTP-mediated receptor binding (47) and cross-linking when intracellular Ca^{2+} levels rise (1, 2). The TGase 3 enzyme was expressed to a minor extent in all tissues tested, but more significantly in the amygdala (Fig. 2). Substantial expression of TGase 1 mRNA was observed in normal cerebellum and corpus callosum where it represented 20–25% of the total (Fig. 3). Unlike epithelial tissues where most TGase 1 is membrane bound, however, we found in these brain tissues that only about 20% of the total TGase activity was associated with membranes from the cortex and cerebellum (Fig. 4), all of which was due to the TGase 1 protein (Fig. 5). Thus up to 80% of the TGase 1 enzyme was present in the cytosolic fraction. Because of its relatively low activity levels (41) (Fig. 4), this means that much of it remained in its intact low specific activity (unprocessed) form. Accordingly, future studies should focus on the biochemical properties of the TGase 1 enzyme and its complex processed forms in brain tissues.

To address the issue as to whether the TGase 1, 2, and 3 enzymes are in fact functional in brain tissues, we isolated and quantitated the isopeptide cross-link formed by these enzymes. Using total tissue sections, we recovered only traces of the cross-link from normal cortex and cerebellum (~1 residue/10,000 residues). This amount is similar to that found in a variety of normal tissues such as liver and muscle (1–4). However, further work will be necessary to identify the natural substrates of the TGases in normal brain tissues.

**Analysis of TGase Expression and Function in AD**—However, in AD cortex and cerebellum tissues, we found 30–50-fold larger amounts of the cross-link, and reflecting this large increase, we were able to isolate highly insoluble proteins following exhaustive extraction in SDS-dithiothreitol solutions. To the best of our knowledge, this is the first report of the direct isolation of the cross-link from AD patient material. Proteins cross-linked to this extent in in vitro assays form large insoluble macromolecular aggregates (1), and insoluble aggregates formed in natural processes such as apoptosis are toxic for cells (4, 6, 48). By extrapolation therefore, it is to be expected that such insoluble protein inclusions could contribute to progressive neurodegenerative disease.

Accordingly, in view of the foregoing discussion, a new set of questions arises as to how these enzymes become problematic in AD disease. In this regard, it is known that expression of proteases including calpains are increased abnormally in AD disease (49–51). Although the detailed molecular events involved in proteolytic activation of TGases 1 or 3 in epithelia are not yet known, we have observed in calcium-dependent differentiation of normal human epidermal keratinocytes that calpain may process TGase 1 into the high specific activity forms and that calpain inhibitors interfere with this process (52). Thus the marked increases of TGase activity in both the cytosol and membrane fractions of especially the cerebellum tissue observed here (Fig. 4), raise the possibility that the TGase 1 and 3 enzymes may be proteolytically activated abnormally in AD disease. However, it is less clear why similar changes were not also observed in cortex tissue (Fig. 4), although the simplest explanation at this time is that there are variable degrees of proteolytic activation. Finally, amyloid deposits, neurofibrillary tangles, and accumulations of insoluble paired helical filaments are also features of amygdala tissue in AD disease (53), but we were unable to acquire this tissue to explore the potential role of the TGase 3 enzyme.

Generally similar data for the TGase 2 enzyme in normal and AD brain tissues have been reported earlier (29). However, in contrast to our study, they noted modest increases of TGase 2 protein and activity in the cortex but little change in the cerebellum between normal and AD patients. As they examined only the cytosolic fraction, such differences in the two studies may be due in part to the presence of significant amounts of TGase 1 enzyme activity in the cytosolic fraction as well. Another possibility is that minor variations in the post-mortem time of the tissues collected may potentially result in proteolytic activation of the cytosolic TGase 1 enzyme but loss of some TGase 2 enzyme.

**Conclusion**—A recent report has suggested that the primary cause of Huntington’s disease may be TGase-induced cross-linking/polymerization of the protein Huntingtin which has an abnormally expanded polyglutamine tract (54), and furthermore, this polymerization could be inhibited by the TGase inhibitors. These data suggest that TGases may be involved in the downstream events of neuronal degenerative diseases such as Huntington’s disease and AD. In this study, we have demonstrated that not only the TGase 2 but also the TGases 1 and 3 enzymes are present and differentially expressed in normal adult human brain tissues. Furthermore, we show that the TGase 1 and 2 enzymes are significantly up-regulated in AD disease. Moreover, the direct isolation and quantitation of the isopeptide cross-link from insoluble protein inclusions from AD tissues affords a robust causal relationship between aberrantly increased TGase activity and disease. Further studies of these
TGases seem desirable to better understand the pathogenesis of neuronal disease.

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