Special Issue

In vitro neurotoxicity of amyloid β -peptide cross-linked by transglutaminase

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Abstract

Transglutaminase catalyzes the intermolecular cross-linking of peptides between Gln and Lys residues, forming an ϵ -(γ -glutamyl) lysine bond. Amyloid β -peptide, a major constituent of the deposits in Alzheimer disease, contains Lys16, Lys28, and Gln15 which may act as substrates of transglutaminase. Transglutaminase treatment of amyloid β -peptide (1–28) and amyloid β -peptide (1–40) yielded cross-linked oligomers. Transglutaminase-treated A β retarded neurite extension of PC12 cells, and rat cultured neurons of hippocampus and septum, brain areas severely affected by Alzheimer disease, and subsequently caused cell death, whereas the transglutaminase-untreated counterparts did not show harmful effects. The transglutaminase-catalyzed oligomers of amyloid β -peptide and their neurotoxicity may be involved in two characteristics in Alzheimer disease, neuronal degeneration and formation of the insoluble deposits.

Abbreviations: AD – Alzheimer disease, A β – amyloid β -peptide, DMEM – Dulbecco's modified Eagle's medium, DMEM/F–12–1:1 mixture of DMEM and Ham's F–12 medium, FCS – fetal calf serum, HS – horse serum, PAGE – polyacrylamide gel electrophoresis, MTT – 3-(4,5-dimethylthiazol–2-yl)–2,5-diphenyltetrazolium bromide, NGF – nerve growth factor, TGase – transglutaminase

Introduction

Formation of senile cerebral amyloid plaques containing insoluble extracellular deposits is one of the hallmarks of AD (Glenner, 1988; Selkoe, 1989). Amyloid β -peptide, a major constituent of the deposits, is a 40–43 amino acid peptide derived from a large membrane-bound amyloid precursor protein (Kang *et al.*, 1987; Dyrks *et al.*, 1988; Esch *et al.*, 1990; Haass and Selkoe, 1993; Sisoda and Price, 1995) and has been thought to play an active role in genesis of AD (Glenner and Wong, 1984; Mattson *et al.*, 1993; Mullan and Crawford, 1993; Selkoe, 1993; Games *et al.*, 1995). This peptide and its fragments form *in vitro* self-aggregates and the *in vitro* neurotoxicity of these peptides appears to be closely correlated with their aggregation state (Kirschner *et al.*, 1987; Yankner *et*

al., 1990; Pike *et al.*, 1991; Burdick *et al.*, 1992; Pike *et al.*, 1993; Lorenzo and Yankner, 1994; Ueda *et al.*, 1994). But the finding that A β is constitutively produced in the normal metabolism of amyloid precursor protein (Haass *et al.*, 1992; Seubert *et al.*, 1992; Shoji *et al.*, 1992) indicates that A β modifications other than the noncovalent self-aggregation may also operate on the formation of the insoluble deposits. The insoluble characteristics of amyloid cores suggest the occurrence of covalent bonds (Smith *et al.*, 1994). Two pathways leading to the formation of covalently bonded cross-linkages have been proposed (Hensley *et al.*, 1994; Smith *et al.*, 1994; Vitek *et al.*, 1994). A β was shown to fragment in aqueous solution, generating cytotoxic free radical peptides, which would also form cross-linked peptide aggregates (Hensley *et al.*, 1994). Glycation of proteins termed Maillard reaction

is initiated by the condensation between amino groups of proteins and reducing sugars, resulting in the formation of advanced Maillard reaction end products which contain proteins cross-links, and are highly insoluble and resistant to proteases. The presence of these end products was found immunocytochemically in senile plaques in brain tissue from patients with AD (Smith *et al.*, 1994). It was shown that the *in vitro* Maillard reaction products of A β acted as a seed for the accelerated aggregation of A β , and that plaque fractions of AD brains contained more advanced Maillard reaction end products than did the preparations from controls (Vitek *et al.*, 1994).

TGase catalyzes intermolecular cross-linking of peptides between Gln and Lys residues, forming ϵ -(γ -glutamyl) lysine bond. This bond is stable and resistant to proteolysis, thereby giving the resistance of the cross-linked products to enzymatic degradation. TGase occurs in plasma, tissues and extracellular fluids, and calcium is a major activator of the activity (Lorand and Conrad, 1984; Greenberg *et al.*, 1991). Treatment of A β with TGase has been shown to produce cross-linked products in which Gln15 in one A β and Lys16 or 28 in another are cross-linked (Dudek and Johnson, 1994; Ikura *et al.*, 1994), but effects of these products on neurons have not been examined. Here we report that TGase-catalyzed cross-linked A β s are toxic to the cultured neuronal cells under the conditions where the TGase-untreated peptides are not toxic. TGase was shown to be present in nerve terminals and implicated in the regulation of exocytosis of synaptic vesicles (Facchiano *et al.*, 1993). Formation of the neurotoxic peptides through TGase reaction may be another route to connect the two features, formation of the insoluble deposits and neurodegeneration, in AD.

Materials and methods

Cross-linking of A β by TGase

TGase was isolated from guinea pig liver (Ikura *et al.*, 1985) and prepared in 10 mM Hepes, pH 7.5, 2 mM EDTA, 0.2 mM dithiothreitol. TGase was pretreated with 5 mM dithiothreitol at 37 °C for 30 min for activation. The reaction mixture for TGase-catalyzed cross-linking of A β (1–28) or A β (1–40) contained 50 μ g ml⁻¹ TGase, 15 mM Hepes, pH 7.5, 1.5 mM dithiothreitol, 600 μ M synthetic A β (Peptide Research Institute in Osaka, Japan), and 5 mM CaCl₂. The reaction was started by the addition of CaCl₂ and incubated

at 37 °C. Controls were run without TGase or A β . A β solutions were freshly prepared just before experiments. It was impossible to separate A β from other components in the TGase reaction mixture. To assay effects of TGase-treated A β on the cells, therefore, the mixture was directly added to the cell cultures. Before addition, the mixture was diluted by the addition of the assay medium described below.

Cell culture

Cells were cultured under a humidified atmosphere containing 5% CO₂ at 37 °C. PC12 cells were maintained in DMEM supplemented with 10% HS and 5% FCS. The cells were pretreated with 100 ng ml⁻¹ NGF for 3 days before cultures for experiments; the pretreatment sensitized the cell response to the re-addition of NGF with respect to the neurite outgrowth. For cultures of experiments, PC12 cells were collected by trypsinization and subsequent centrifugation, and then the cells were suspended in DMEM containing 10% HS, 5% FCS and 100 ng ml⁻¹ NGF. Hippocampal and septum neurons were prepared from embryonic day 18 Wistar rats. The brain was removed from fetal rats under sterile condition. Hippocampus and septum areas were dissected under a stereomicroscope with a fiber optic light source and treated with 0.25% trypsin in 10 mM Na-phosphate buffered saline, pH 7.4, at 37 °C for 10 min. The reaction was stopped by addition of an equal volume of 1:1 mixture of DMEM and DMEM/F-12 containing 5% HS, 5% FCS, 15 mM Hepes, pH 7.5, and 0.01% DNase I. After mechanical dissociation by pipetting, the neurons were pelleted by centrifugation and suspended in DMEM/F-12 containing 5% HS, 5% FCS and 15 mM Hepes, pH 7.5. The cells were filtered through a lens paper. PC12 cells, hippocampal and septum cells were plated on 10 mg ml⁻¹ poly-L-lysine coated 96-well plate at a cell density of 1 $\times$ 10⁴, 6 $\times$ 10⁴ and 1.5 $\times$ 10⁵ cells per well, respectively. After culture for 24 h, the medium was replaced with the new medium to assay the effects of A β on the cells. The assay medium consisted of DMEM/F-12, GMS-X supplement (Gibco BRL), 0.8 μ M progesterone, 1 μ M DL- α -tocopherol, 15 mM Hepes, pH 7.5, and a sample to be assayed. In the case of PC12 cells, the assay medium contained 100 ng ml⁻¹ NGF.

Electrophoresis and MTT assay

SDS-PAGE was done as described by Laemmli

Figure 1. SDS-PAGE of TGase-treated A β (1–40). Lane 1, molecular mass marker proteins; lane 2, A β monomer; lane 3, A β incubated for 180 min in the TGase reaction mixture containing 36 mM EDTA; lane 4, A β treated with TGase for 20 min; lane 5, A β treated with TGase for 180 min. Lanes 2 to 5 contained 3.6 μ g of the peptide. SDS-PAGE was done using polyacrylamide gradient (15 to 25%) gels. Samples were added to reducing buffer, heated for 3 min at 100 °C, and electrophoresed. The gels were stained with 0.2% Coomassie brilliant blue.

(Laemmli, 1970) using polyacrylamide gradient (15 to 25%) gels. Samples were added to reducing buffer, heated for 3 min at 100 °C, and electrophoresed. The gels were stained with 0.2% Coomassie brilliant blue containing 40% methanol and 10% acetic acid. To assess A β cytotoxicity, cell survival was assayed colorimetrically using the cleavage of MTT by living cells as described previously (Mosmann, 1983).

Results

Cross-linking of A β by TGase

Figure 1 shows TGase-catalyzed oligomerization of

A β (1–40). The monomeric form of the peptide decreased upon incubation with TGase, accompanying with concomitant appearance of oligomeric forms (lanes 3 and 4). As judged by migration, dimer and tetramer appear to be major products. When the peptide was analyzed with SDS-PAGE without incubation (lane 1) or after incubation for 180 min in the presence of EDTA, an inhibitor of Ca²⁺-dependent TGase (lane 2), no oligomers were detected. Similar results were obtained with A β (1–28), consistent with the results in our previous paper (Ikura *et al.*, 1994).

It has been shown that A β -derived peptides containing Met35 generates free radicals, which would cause the polymer formation, and that Hepes retards the free radical production (Hensley *et al.*, 1994). To repress the free radical production, in the present paper we used Hepes buffer for TGase reactions. Others who carried out TGase reactions in Tris buffer using A β (1–40) as a substrate, described formation of high-molecular weight polymers including those incapable of entering into the separating gels (Dudek and Johnson, 1994). These polymers were not formed when the reactions were done in Hepes buffer (Figure 1). The free radical-mediated A β (1–40) oligomers in Tris buffer, even though they were formed in a very small amount, might act as cores for TGase reaction, resulting in the detectable amounts of high-molecular weight polymers as seen in the previous paper (Dudek and Johnson, 1994). For technical reason that it was impossible to separate A β from other components in the TGase reaction mixture, the mixture was added directly to the cultured neuronal cells. Hepes showed no effects on the cells, while Tris was toxic (data not shown). Thus, in our system effects of TGase-catalyzed products of A β can be assessed.

Toxicity of cross-linked A β on PC12 cells

Cells derived from the neural crest lineage appear to display in culture either neuronal or chromaffin phenotypes. NGF induces neuronal characteristics and corticosteroids potentiate chromaffin properties. The rat cell line PC12, which has been established from an adrenal medullary pheochromocytoma and has been widely used as a model system of neurons, responds to treatment with NGF by extending neuronal-like processes (Green and Tischler, 1976). PC12 cells were precultured for 24 h and then the culture for testing effects of A β on the cells was started by replacing the medium with the fresh medium containing NGF and the sample tested. During further culture of the cells,

we found that TGase-catalyzed cross-linked A β had deteriorative effects on the cells. Extension of neuritic processes is evident in the cells cultured for 1 day in the presence of NGF alone (Figure 2A), while a very few processes with short length were formed in the presence of NGF and TGase-catalyzed cross-linked A β (1–40) (Figure 2D). Cultures with the vehicle solutions containing the TGase reaction mixtures without TGase or A β (1–40) showed little repression of NGF-induced neurite outgrowth (Figures 2B and C). In addition to the repressive effect on induction of the neuronal property by NGF, cross-linked A β degenerated the cells; MTT assay indicated that most of the cells died upon culture for 3 days but that the cells in all control cultures survived (data not shown). Similar results were obtained with A β (1–28). The free radical scavenger vitamin E, which inhibited A β induced-neuronal cell death (Behl *et al.*, 1992), did not show protective effect, suggesting that the deleterious effects of TGase-treated A β are not due to the free radicals. This view is supported by the finding that the TGase-treated A β (1–28) shows the similar effects, although A β (1–28) does not contain Met35, which is thought to be a key amino acid for A β radicalization (Hensley *et al.*, 1994). Preculture of the cells with NGF reduced deteriorative potency of the cross-linked A β depending on the length of preculture. From microscopic observation, it appeared that addition of cross-linked A β to the cells precultured with NGF for 3 days did not give significant effects on neurites and cell survival.

Toxicity of cross-linked A β on primary cultured neurons

Neurons from hippocampus or septum of embryonal day 18 rats were precultured for 24 h and then the cells were cultured to examine the effects of TGase-catalyzed products. Figure 3 shows the results of hippocampal neurons. Marked toxicity of TGase-treated A β (1–40) was seen in the culture for 24 h. Extension of neurites was severely inhibited and the cells were degenerated (Figure 3D), while there were little harmful effects when the cells were cultured in the presence of vehicle solutions that contained the TGase-reaction mixture without TGase or A β (Figures 3B and C). These effects were also seen with septum neurons (data not shown).

Neurons were cultured for 3 days with cross-linked A β (1–40) and their cytotoxicity was estimated quantitatively with MTT assay. As shown in Figure 4, the cytotoxic effect on hippocampal and septum neurons

was dose-dependent. Concentrations of A β are represented as monomer concentrations. From rough estimation of the cross-linked products (see Figure 1), the concentrations of cross-linked products with neurotoxicity appear to be less than 10% of the monomer concentrations. The vehicle solution containing A β but no TGase showed no effects, indicating that A β monomer was not cytotoxic under culture conditions that we used. Similarly, TGase-treated A β (1–28) repressed neurite extension and caused cell death, whereas the TGase-untreated A β (1–28) did not (data not shown). As seen with PC12 cells, TGase-catalyzed cross-linked A β s were less toxic to the neurons precultured; addition of the cross-linked A β to the neurons precultured for 6 days did not show the deteriorative effects.

We did not examine TGase-catalyzed cross-linked products of scrambled peptides that were equivalent to A β (1–28) and A β (1–40) in amino acid compositions but different in their sequences. Neither synthetic ϵ -(γ -glutamyl) lysine nor TGase-catalyzed oligomers of parathyroid hormone (84 amino acid residues including three Lys and two Gln residues) was toxic to the cultured neurons.

Discussion

A β (1–28) corresponds to the extracellular portion of A β and A β (1–42) contains a substantial portion of A β transmembrane region (Gly29-Leu52). Various synthetic A β s form filamentous β -sheet assemblies *in vitro* but only A β s containing a substantial portion of transmembrane region form stable aggregates (Burdick *et al.*, 1992). Formation of the aggregates is dependent on pH, A β concentrations, and incubation time (Burdick *et al.*, 1992). Freshly prepared A β s are not toxic to the cultured neurons and the toxicity appears to be exhibited by these aggregates (Pike *et al.*, 1991; Pike *et al.*, 1993; Ueda *et al.*, 1994). It was reported that preincubation of A β (1–42) yielded aggregates with toxicity, whereas preincubation of A β (1–28) resulted in neither stable aggregation formation nor toxicity (Pike *et al.*, 1991; Pike *et al.*, 1993). From comparison of the preincubation conditions with TGase reaction conditions used in this paper, formation of A β (1–40) aggregates during TGase reaction is unlikely. In fact, A β (1–40) incubated without TGase lacks toxicity. Taken together with our finding that TGase-treated A β (1–28) is toxic but the A β -unrelated peptide containing TGase-catalyzed isopeptide bonds is not, it can be speculated that an A β specific sequence surrounding the isopep-

Figure 2. Toxic effect of TGase-catalyzed cross-linked A β (1–40) on PC12 cells. The cells were cultured for 24 h in the presence of 100 ng ml⁻¹ NGF and the following samples. (A), none; (B), TGase reaction mixture incubated for 180 min in the absence of A β ; (C), TGase reaction mixture incubated for 180 min in the presence of A β but without TGase; (D), complete TGase reaction mixture incubated for 180 min. A β was added to the cell cultures at the concentration of 40 μ M. Scale bar, 10 μ m.

tide bonds formed by TGase treatment plays a crucial role to exhibit neurotoxicity *in vitro*.

In some rare genetically linked familial AD, formation of amyloid plaques may be related with an increased level of soluble A β (Citron *et al.*, 1992; Cai *et al.*, 1993) but it appears that this relation can not be applied in sporadic cases (Shoji *et al.*, 1992). Then it is important to identify factors that may contribute to an increase in rate of amyloid formation or a decrease in rate of amyloid degradation. Formation of TGase-catalyzed cross-linked A β may contribute to both. The *in vitro* aggregation of synthetic A β proceeds through processes referred to as nucleation-dependent polymerization (Jarett and Lansbury, 1993). Since the nucleus formation is a thermodynamically unfavorable process, at low concentrations of A β monomer the formation of A β aggregates requires monomer concentration-dependent lag period during which little

aggregates are formed. Once the nucleus has formed, its growth becomes more rapid because monomers bind with the multiple interaction sites exposed on the nucleus. Thus the nucleation of A β monomers can be a rate-limiting step of amyloid plaque formation. Although we have not examined potency of TGase-catalyzed cross-linked A β as the nucleus for further aggregation in a quantitative manner, large macroscopically recognizable aggregates have been formed upon long incubation of TGase reaction mixture but not formed without TGase, which suggests that the A β oligomers function as seed material for aggregate formation (unpublished data). Formation of protein cross-links by TGase generally provides higher resistance to protease digestion due to stabilized protein structure or loss of substrate sites (Lorand and Conrad, 1984; Greenberg *et al.*, 1991); cross-linking of A β by TGase may result in a decreased rate of the *in vivo* degrada-

Figure 3. Toxic effect of TGase-catalyzed cross-linked A β (1–40) on cultured hippocampal neurons. The cells were cultured for 24 h in the presence of the following samples. (A), none; (B), TGase reaction mixture incubated for 180 min in the absence of A β ; (C), TGase reaction mixture incubated for 180 min in the presence of A β but without TGase; (D), complete TGase reaction mixture incubated for 180 min. A β was added to the cell cultures at the concentration of 40 μ M. Scale bar, 10 μ m.

tion of A β , thereby accelerating formation of amyloid plaques.

Where does TGase encounter with A β ? Although accumulation of A β aggregates in lysosomes has been proposed (Knauer *et al.*, 1992), the presence of soluble A β in cerebrospinal fluid indicates that the extracellular accumulation can also occur. Tissue-type TGase is mainly regulated by interplay between inhibition by GTP and activation by calcium (Greenberg *et al.*, 1991). TGase isolated from rat brain showed these characteristics (Takeuchi, H. personal communication). Two isoforms of TGase are present in brain nerve endings; one is bound to synaptic vesicles and the other is in cytosolic compartment (Facchiano *et al.*, 1993). As a physiological role of TGase in neurons, it has been hypothesized that TGase activated by neurotransmitter-induced calcium entry inhibits exocytosis of synaptic vesicles, resulting in repression

of excessive release of neurotransmitter (Facchiano *et al.*, 1993). TGase may leak from synaptic terminals compromised by a brain insult to extracellular sites where calcium concentration is high enough to fully activate TGase. The leaked TGase, even though the leakage is very fractional, produces cross-linked A β using extracellular soluble A β as substrate, which in turn are neurotoxic and may be 'seed' for amyloid plaque formation. The repressive effect of TGase-catalyzed A β products on neurite extension of cultured neuronal cells suggests that these products interfere with reconstitution of the injured synapses, leading to the accelerated leak of TGase. Further studies to verify this hypothesis requires demonstration of the presence of TGase-catalyzed isopeptide bonds in amyloid plaques, although TGase-catalyzed cross-links may not be necessarily produced at detectable

Figure 4. Toxicity of TGase-catalyzed cross-linked A β (1-40) on cultured neurons. To assess A β cytotoxicity, cell survival was assayed colorimetrically using the cleavage of MTT. (A), hippocampal neurons, (B) septum neurons. The cells were cultured for 3 days in the presence of the following samples and cell survival was assessed by the MTT assay. ($\circ$), TGase reaction mixture incubated for 180 min in the absence of TGase; ($\bullet$), complete TGase reaction mixture incubated for 180 min. A β concentrations are expressed as monomer concentrations. From rough estimation of the cross-linked products (see Figure 1), the true concentrations of cross-linked products with neurotoxicity are less than 10% of the total monomer concentrations. The values are the mean $\pm$ SEM for triplicate assays.

levels because the cross-linked A β may function as the nucleus for amyloid plaque formation.

A β (1–42)/A β (1–43) has been reported to be the A β predominant species in senile plaques (Iwatsubo *et al.*, 1994) and these species nucleate rapidly into amyloid fibrils (Jarrett and Lansbury, 1993). TGase-catalyzed cross-links of these peptides may enhance the nucleation.

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