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TRANSGLUTAMINASE AND DISEASES OF THE CENTRAL NERVOUS SYSTEM

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1. ABSTRACT

Alzheimer's disease, Parkinson's disease and diseases of expanded polyglutamine are associated with insoluble protein aggregates and neuronal death. A role for transglutaminase in the stabilization of these aggregates has been proposed. Diseases of polyglutamine expansion have been the most thoroughly investigated and a large body of studies supports the causative role of transglutaminase in aggregation of expanded polyglutamine. However none of the evidence is conclusive. Indisputable evidence of cross-linking by transglutaminase will be required in order to provide firm support for therapeutic measures based on the role of transglutaminase.

2. INTRODUCTION

Transglutaminase catalyzes the formation of aggregates by introducing intermolecular isopeptide cross-links between glutamine and lysine residues of polypeptides. A number of human diseases of the central nervous system are characteristically associated with protein aggregates and neuronal death. These diseases include Alzheimer's disease, Parkinson's disease and diseases of expanded polyglutamine. The relation between aggregate formation in diseases of polyQ expansion and transglutaminase has been extensively studied. This review will therefore concentrate on diseases of polyQ expansion and deal more briefly with Alzheimer's and Parkinson's disease.

A clear distinction must be drawn between the diseases of polyQ expansion and the other neuronal diseases associated with protein aggregation, such as Alzheimer's and Parkinson's disease. Diseases of polyQ expansion are each caused by the presence of an abnormally long polyQ sequence within a specific protein. Because the glutamine residue is necessary for the formation of the isopeptide epsilon-(gamma-glutamyl) lysine cross-links, it has been proposed that the protein bearing the expanded polyQ becomes a substrate for transglutaminase, thus generating aggregates that kill neurons (1). The main forms of Alzheimer's and Parkinson's disease are not genetically transmitted and are not associated with a protein that is modified in such a way as to make it a better substrate of transglutaminase.

Transglutaminase is found in all parts of the brain (2). Neurons possess three transglutaminases (numbered 1-3), of which transglutaminase 2 (tissue type) is the most abundant (3). In addition to its ability to form cross-links when activated by Ca^{2+}, transglutaminase 2 has been shown to function as a signal transducing G protein with guanosine triphosphatase activity (4). This latter activity is probably irrelevant to neurological diseases. It has been proposed that the cross-linking activity of transglutaminase was related to cell death, whether programmed or due to toxic agents (5). However, targeted inactivation of transglutaminase 2 does not produce any obvious phenotype (6). Absence of any function or compensation by the other transglutaminases can explain the lack of phenotype. In most cell types, cellular transglutaminase is latent, since the Ca^{2+} concentration of the cells (10^{-6} M) is too low to activate the enzyme. In cell death, cytosolic concentration of Ca^{2+} is likely to rise, owing to its liberation from the mitochondria or the endoplasmic reticulum, or to loss of the plasma membrane barrier. For this reason, transglutaminase activation might be the consequence of cell death rather than its cause.

The presence of isopeptide cross-links in normal brain shows that brain transglutaminase can be activated (7). In the case of neurons, transient rises in Ca^{2+} are physiological. Calculations have led to the conclusion that the Ca^{2+} increase in the cytoplasmic zone up to 10 nm from the pore of an open Ca^{2+} channel may bring the Ca^{2+} concentration up to a value three orders of magnitude higher than in the rest of the cytosol (8), or at the optimal level for transglutaminase. This might explain why the inclusions associated with the diseases of polyQ expansion are found only in neurons, although the mutant proteins are present in a large number of unaffected cell types.

3. DISEASES OF POLYGLUTAMINE EXPANSION

3.1. Diseases of polyglutamine expansion share a common pathogenic mechanism

There are nine diseases of the human central nervous system, each associated with a different protein containing an expanded polyglutamine sequence: spinal and bulbar muscular atrophy (SBMA) (9), Huntington disease (10), dentatorubral-pallidoluysian atrophy (DRPLA) (11), spinocerebellar ataxias (SCAs) types 1 (12), 2 (13-15), 3 (16), 6 (17), and 7 (18). The presence of a TATA binding protein containing an expanded polyQ is a very rare cause of the autosomal dominant cerebellar ataxia SCA17 (19, 20). The implication of a CAG repeat expansion in autosomal dominant pure spastic paraplegia (21) has been challenged (22). The most frequent of these diseases is Huntington disease: there are about 25,000 cases and 100,000 people at risk in the United States. In the causative proteins of all nine diseases, there is normally a polyglutamine sequence of about 20 glutamine residues. When the number exceeds some higher value (35 in some proteins, as high as 54 in others) the protein produces disease of the central nervous system (23). Like expansion of microsatellites in intergenic DNA (24), expansion of encoding polyCAG is thought to occur by slipped strand mispairing/hairpin formation, during replication.

The nine diseases share a number of properties: 1) autosomal dominant inheritance, with the exception of SBMA, which is linked to the X chromosome; 2) anticipation, which means that the disease tends to begin earlier and to become more severe with successive generations in affected families, generally when the expanded allele is transmitted by the father; 3) the severity of the disease is closely correlated with the polyglutamine length; 4) commonly late onset: for instance, the mean age of onset for Huntington disease is 38 years; 5) all diseases affect the central nervous system, although the proteins are generally present in similar amounts in a large variety of unaffected tissues. The fact that the diseases of expanded polyglutamine share so many specific properties argues strongly for a common pathogenic mechanism.

3.2. Expanded polyglutamine produces dominant gain of function by neuronal proteins

Dominant inheritance can be due to gain of function, dominant negative mutation or haploinsufficiency, but there are a number of arguments favoring the gain of function hypothesis. The absence of one huntingtin allele does not produce Huntington disease (25). A transgene encoding only a small fragment of huntingtin containing expanded polyglutamine produces disease of the central nervous system. It seems implausible that such a fragment could have retained enough of the function of the protein to act as a dominant negative; in addition, haploinsufficiency is ruled out since the transgene is accompanied by the two normal huntingtin alleles (26). A short fragment of the SCA3 protein bearing an expanded polyglutamine sequence also causes neurological disease in mice (27). A polyglutamine sequence inserted in a protein totally unrelated to any of the disease-causing proteins, the enzyme hypoxanthine phosphoribosyl transferase (HPRT), produces disease of the nervous system in mice (28). Disruption of the two huntingtin alleles in mice causes death of the embryos at gastrulation, but patients homozygous for Huntington disease alleles have no developmental defects and have no more serious disease than heterozygotes (29). The disease must be the consequence of a protein alteration that does not affect its normal function. Deletion of the androgen receptor gene results in testicular feminization in males because of androgen insensitivity, but does not produce central

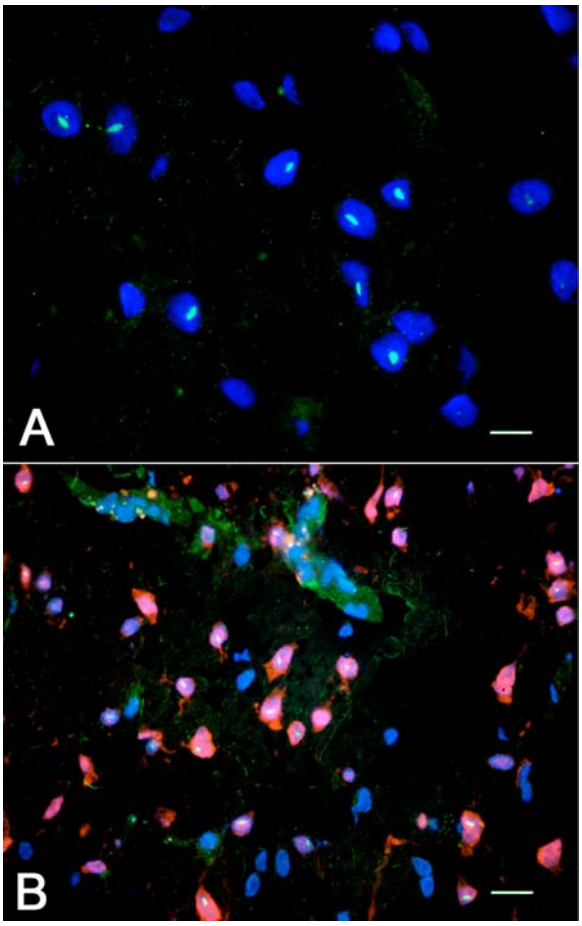

Figure 1. Inclusions in brain of patients with Huntington disease. Frozen sections were stained with an antibody directed against the N-terminus of huntingtin (green) and the nuclei counterstained with Hoechst 33258. (A) In a juvenile case, staining of Brodmann area 7 of the cerebral cortex with the anti-huntingtin antibody reveals the presence of numerous nuclear inclusions. Bar graph: 10 μm. (B) Double staining of a protein specific to neuronal nuclei, NeuN (red) shows that virtually all inclusions reside in neurons. Bar graph: 20 μm. Reproduced from (54) with permission from Biochimie.

nervous system disease (30). Expansion of the polyglutamine sequence results in SBMA, together with mild symptoms of androgen insensitivity. In this disease, polyglutamine expansion causes both a gain of function and a partial loss of function (31), but the neurological disease is due to the gain of function.

3.3. The gain of function produced by expanded polyglutamine in neuronal proteins results in cell lethality

Extensive neuronal loss has been observed in the regions of the brain affected by the various diseases of expanded polyglutamine. For instance, there is a large decrease in neuronal density within the caudate and cortex of patients with Huntington disease (32), whereas no neuronal loss is observed in unaffected regions, such as the cerebellum (33, 34). Progressive neuronal loss has been observed in transgenic mice and *Drosophila* expressing an expanded polyglutamine (27, 35, 36). There is a direct correlation between the extent of neuronal loss and the severity of the disease: up to 95 % of the neurons of the caudate are lost in grade 4 patients, but only one third in more mildly affected cases (37). In humans or transgenic animals, toxicity of the protein with expanded polyglutamine appears to be confined to neurons, in spite of an ubiquitous distribution of the proteins; but the growth of COS cells, a non-neuronal cell type, is inhibited by expression of a cDNA encoding a fragment of the ataxin 3 containing an expanded polyglutamine (27). Apoptotic cell death has been observed in cells transfected with a cDNA encoding truncated DRPLA protein or huntingtin with an expanded polyglutamine, while similar cDNAs containing normal-length polyglutamine had no effect (38, 39). Toxicity for E. coli of a glutathione-S transferase (GST) fusion protein containing a long polyglutamine sequence has been reported (40).

3.4. Protein aggregation as the cause of cell lethality

Aggregates or inclusions containing the protein with expanded polyglutamine have been described in regions of the brain affected by Huntington disease (41, 42), SCA1 (43), SCA2 (44) SCA3 (45), DRPLA (46), SCA7 (47), SCA6 (48) and SCA17 (49-50). Nuclear inclusions found in the cortex of a patient with juvenile Huntington disease are illustrated in Figure 1. A large body of literature supports the view that aggregates are the cause of neuronal death. The usually late onset of the diseases and their slow progressive nature are most easily explained by a slow aggregation process in which the aggregates become lethal beyond a certain amount.

Here is a partial list of the arguments for a causative role of the aggregates. There is a direct correlation between frequency of inclusions in neurons and severity of Huntington disease (51, 52). In a pre-symptomatic person who carried an expanded huntingtin gene and who died of an accidental cause, no inclusions were detected in either the cortex or the striatum (42), whereas all patients who died of Huntington disease possessed inclusions. Patients with juvenile Huntington disease who have large numbers of inclusions in their cortical neurons, have none in either their cerebellum or their globus pallidus, both of which are spared by the disease (53, 54). The predicted lag times for polyQ aggregation derived from *in vitro* data correlate with the age of onset/CAG repeat curves seen in Huntington disease (55, 56). In transgenic mice, inclusions precede the disease (41). Inclusions formed in cultured cells correlate with susceptibility to cell death (51, 45, 38, 57-59). In striatal neurons, aggregates cause neuritic degeneration before nuclear fragmentation (60). Reduction of inclusion formation by over-expression of heat shock proteins (HSPs) and chaperones is associated with decreased cell death (61-63). Monoclonal antibodies that inhibited aggregation of mutant huntingtin in cultured cells also inhibited cell death, whereas two monoclonal antibodies that stimulated aggregation increased cell death (64). A similar correlation between aggregation and cell death has

been found in cultured cells, using suppressor peptides (65) and using Congo red (66). A strong correlation between formation of aggregates and cell death has been observed in *Drosophila* (65). Aggregates of polyglutamine formed *in vitro* and introduced into cultured cells produce cell death (67). When extracts of the most affected regions of the brain of patients with Huntington disease were submitted to electrophoresis and immunoblotting, the discrete band of huntingtin containing the expanded polyglutamine was replaced by a broad band extending into the region of larger molecular weight, while the band of normal huntingtin was unchanged. Since extracts of most cell types or of relatively spared regions of the central nervous system contain the two forms of huntingtin in nearly equal amounts, there appears to be specifically reduced mobility of huntingtin bearing the expanded polyglutamine in the most affected regions of the brain (53, 68, 69). Smearing does not result from mosaicism of the expanded allele within the cortex and must therefore be the consequence of aggregation (70). It has been demonstrated recently that expanded polyglutamine is incorporated into oligomers and polymers in the cortical neurons, but not in the cerebellar neurons of Huntington disease patients (71).

A number of papers have argued that nuclear inclusions are not the cause of cell lethality (72-74). By various means, these investigators found absence of correlation between the presence of nuclear inclusions and cell death. This evidence applies only to microscopically visible aggregates. As described above, numerous authors have shown a correlation between the presence of molecular aggregates of huntingtin and the regions of the brain damaged in Huntington disease. Moreover, nearly all monomeric expanded huntingtin of affected brain was replaced by oligomeric or polymeric huntingtin. This must mean that nearly all cells are affected, whereas inclusions are seen with variable frequency. We conclude that molecular aggregates are characteristic of huntingtin with expanded polyglutamine, whether they make microscopically detectable inclusions or not.

3.5. Two mechanisms of protein aggregation have been proposed

3.5.1. Polar zipper formation

It has been proposed that a protein bearing an expanded polyglutamine can form aggregates stabilized by hydrogen-bonded polar zippers (75). A short polyglutamine sequence (Q_{10}) incorporated into a small protein resulted in the formation of dimers and trimers (76). A purified fragment of huntingtin containing 51 glutamines and a total of 120 amino acids has been shown to form insoluble filamentous aggregates *in vitro* (77).

The first evidence for polar-zipper formation by polyQ sequences in cultured cells came from Hazeki *et al.* (78). They showed that COS cells expressing the exon 1 of huntingtin, encoding an expanded polyQ, accumulated large aggregates, which could be dissolved by treatment with concentrated formic acid and mostly reduced to monomer. Concentrated formic acid is an extremely effective solvent for otherwise insoluble proteins (79). It was later shown that the perinuclear inclusions formed in PC12 cells by an expanded polyQ flanked by the residues adjacent to the polyQ-containing region of the androgen receptor were mostly reduced to monomer after treatment with 96% formic acid. After incubation in formic acid, there remained in both the COS cells and PC12 cells an oligomer, whose resistance to formic acid suggested the participation of covalent bonds in its stabilization (71). These results clearly show that non-covalent bonds, presumably polar-zippers, stabilize the inclusions in cultured cells. The presence of oligomers insoluble in formic acid suggests that isopeptide bonds are also present (see below).

No direct evidence has yet been provided as to the existence of polar zippers in the aggregates or inclusions present in the brain of patients. Hydrogen-bonded beta pleated sheets are known to exhibit green birefringence under polarized light after Congo red staining. Aggregates formed by a pure peptide containing an expanded polyQ sequence also display such a green birefringence (77). The fact that some of the inclusions found in the brain of patients with Huntington's disease were birefringent after staining with Congo red has been taken as evidence that these inclusions contained beta pleated sheets stabilized by hydrogen bonds (80). But others have been unable to stain the inclusions of patients with Congo red (81) and McGowan *et al.* (82) have reported that the inclusions present in exon 1 transgenic mice cannot be stained with Congo red.

3.5.2. Transglutaminase-catalyzed cross-linking

A number of years ago, the hypothesis was advanced that the protein inclusions in the neurons of humans with Huntington disease and other diseases of expanded polyglutamine were stabilized by isopeptide cross-links introduced by transglutaminase. It was postulated that, as a result of excessive glutamine reiteration, the mutant proteins would become substrates of transglutaminase (1).

3.6. Transglutaminase activity in Huntington disease

Tissue transglutaminase is predominantly cytoplasmic, but the inclusions associated with Huntington disease are frequently nuclear, particularly in the juvenile forms of the disease (42, 54). Although human neuroblastoma nuclei contain only 7% of the total transglutaminase of the cell, the nuclear enzyme accounts for about half the total enzyme activity of the cell. When intracellular Ca^{2+} levels are increased, some active transglutaminase appears to translocate to the nucleus (83).

Transglutaminase activity of cortex and striatum has been found to be increased by about two to four fold in Huntington disease, compared to controls (81, 84, 85). In one study, transglutaminase activity of affected brain regions increased with the grade of the disease (84). The results in cerebellum were contradictory. Lesort *et al.* (84) found no increase in cerebellum, compared to controls, whereas Karpuj *et al.* (81) measured a four-fold increase. Results in lymphocytes of patients with Huntington disease were either higher (86) or lower than in normal controls (81).

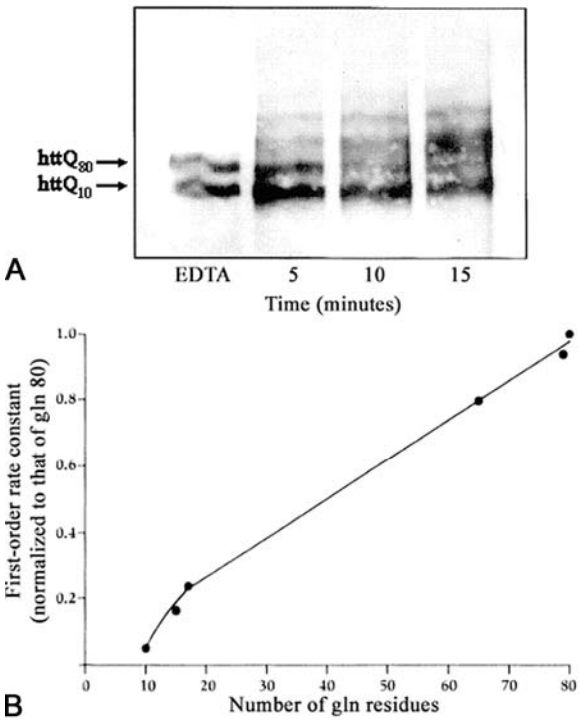

Figure 2. (A) Q80 huntingtin preferred to Q10 huntingtin in transglutaminase-mediated cross-linking. Lymphoblast extract from a lymphoblastoid line containing huntingtins with Q_{10} and Q_{80} was incubated in the presence of tissue transglutaminase. Reaction was stopped by the addition of EDTA, and the products were revealed by immunoblotting. At 10 min, Q_{10} huntingtin was little affected, but Q_{80} huntingtin had nearly disappeared and been replaced by antibody-stained smear and discrete bands extending above the original position of Q_{80} huntingtin; these bands were estimated by extrapolation to be of molecular weights 410, 440, and 480 kDa, compared with 371 kDa for the monomer of expanded huntingtin. The smear extended to about 600 kDa. The control incubated for 15 min in the presence of EDTA shows no loss of Q_{80} huntingtin and no bands or smear of higher molecular weight. (B) Transglutaminase-catalyzed reaction of huntingtins containing polyQs of different lengths after electrophoresis of the reaction products, immunoblotting and chemiluminescence detection, the rate of disappearance of each protein was quantitated by scanning of the films. The figure shows the first-order rate constant increasing with polyglutamine length. Reproduced from (69) with permission from Elsevier.

In summary, it is clear that a significant fraction of transglutaminase activity is in the nucleus, where it can be activated by Ca^{2+} and potentially form nuclear aggregates. It is less clear whether the increased activity observed in cortex and striatum is related to the disease process, since in some cases an increased activity is also found in cerebellum.

3.7. PolyQ-containing peptides as substrates for transglutaminase *in vitro*

As a model for the polyglutamine-induced diseases, work in our laboratory and that of Howard Green showed that a polyglutamine conferred excellent substrate properties on any peptide, as long as the peptides were rendered sufficiently soluble by the flanking residues. Lengthening the polyglutamine increased not only the reactivity of the peptide, but also the reactivity of each glutamine residue. Under optimal conditions, virtually all the Q residues acted as amine acceptors. While externally added transglutaminase increased the cross-linking, brain extract itself possessed sufficient enzyme to form the aggregates (87). In a transglutaminase-catalyzed reaction, Q_{10} and Q_{62} fused to GST were later shown to accept putrescine much more efficiently than did dimethyl casein, a commonly used transglutaminase substrate (88). Under appropriate conditions, GST-Q_{52} incorporates nearly three times more putrescine than GST-Q_{33} (89). Pure polyQs in dilute solution incorporate spermine in proportion to polyQ length (90). These results established that a glutamine repeat, if exposed on the surface of a protein, should form cross-linked aggregates in the presence of any transglutaminase activated by Ca^{2+}.

These experiments were performed on model substrates containing polyglutamine but lacking most of the sequence of the disease-producing protein. It was later shown that full length huntingtin was a substrate of transglutaminase *in vitro* and that the rate constant of the reaction increased strongly with the number of consecutive glutamine residues. As a result, huntingtin with expanded polyglutamine was preferentially incorporated into polymers (Figure 2). In brain regions affected by Huntington disease, the amount of monomeric huntingtin with expanded polyQ was greatly reduced and there appear polymers containing the expanded huntingtin. Therefore transglutaminase action imitated the changes observed in affected parts of the brain (69). N-terminal fragments of huntingtin have been produced *in vitro* by coupled transcription/translation and tested for their ability to form polymers under the action of tissue transglutaminase. An N-terminal fragment with Q_{67} was polymerized to a greater extent than one with Q_{23} (81). N-terminal fragments with Q_{148} have been found to be substrates of the three transglutaminases (types 1-3) present in brain (85).

3.8. Amine donors in cross-linking of expanded polyQ by transglutaminase

When a lymphoblastoid extract containing both normal and expanded huntingtin was incubated in the presence of transglutaminase, the expanded huntingtin became greatly diminished in abundance and was replaced by a smear of huntingtin-containing protein extending above the position of the expanded monomer. Within the smear a few bands were sometimes detected. The presence of a continuous smear showed that expanded huntingtin could be cross-linked to numerous lymphoblastoid proteins, while the presence of a few bands indicated that some lymphoblastoid proteins might be preferred. Similar results were obtained with a brain extract (69). These experiments showed that there must be numerous lysine-donating proteins that could be cross-linked to expanded huntingtin. Even polylysine is a very good amine-donating substrate in vitro (87). It has long been known that there is little specificity in the amine-donating proteins acting as substrates of transglutaminase (91).

A number of proteins have been identified as amine-donors that could be cross-linked to expanded polyQ *in vitro*. The first to be identified were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and alpha-ketoglutarate dehydrogenase. Because the two glycolytic enzymes lost their activity when crosslinked to expanded polyQ, it was suggested that aggregation by transglutaminase lead to neuronal death by disrupting energy metabolism (92). However, this is doubtful since there is no detectable loss in GAPDH activity in the brain of patients with Huntington disease (93). Histone H1, which contains 30% lysine residues, is an excellent substrate of transglutaminase (94). It has been hypothesized that irreversible acylation of histone H1 by covalent attachment of expanded polyQ may cause release of histones from DNA thus deregulating transcription or increasing susceptibility of DNA to DNases (95). No evidence has been provided as to the presence of either GAPDH, alpha-ketoglutarate dehydrogenase or histones in the inclusions found in Huntington disease.

3.9. Effect of overexpression or inhibition of transglutaminase in cultured cells and transgenic mice containing an expanded polyQ

The treatment of cultured cells bearing a truncated DRPLA gene with inhibitors of transglutaminase, such as cystamine, reduced aggregate formation and apoptotic cell death (38). When cultured cells transfected with a construct expressing expanded polyglutamine were also transfected with a construct expressing transglutaminase 2, the frequency of intracellular aggregates was increased, and this frequency was somewhat reduced by treating the cells with cystamine (96). The role of transglutaminase in Huntington disease has been challenged. Human neuroblastoma cells, stably transformed with a transglutaminase 2 antisense construct formed aggregates when they were transiently transfected with an expression vector encoding an N-terminal fragment of huntingtin containing Q_{82}. But it was not clear that these cells had lost all transglutaminase activity, since neurons are thought to contain transglutaminase 1 and transglutaminase 3, in addition to transglutaminase 2 (97).

When the transglutaminase 2 gene in mice was ablated, and the animals were crossed with mice bearing a transgene encoding exon 1 of huntingtin containing 116 CAG repeats (R6/1 and R6/2 mice), the double mutants exhibited amelioration of the course of the disease, compared with the control animals bearing the transgene alone (98, 99). The amount of brain isodipeptide, as measured by phenylisothiocyanate derivatization and high-pressure liquid chromatography, was greatly reduced in the double mutant (98). Both studies reported that the double mutant showed a paradoxical (modest) increase in the frequency of inclusions. This would mean that transglutaminase inhibits the formation of the inclusions. Using an *in vitro* solubility assay, transglutaminase was found to improve the solubility of a thioredoxine-Q_{62} fusion protein, presumably because the intermolecular cross-links introduced by transglutaminase prevented polar-zipper formation (100). A similar inhibitory effect of transglutaminase on the aggregation of amyloidgenic proteins has been reported (101).

The survival of the R6/2 mice was improved by treatment of the animals with cystamine (102, 103). Both papers reported the resulting amelioration of the disease. It should be noted that cystamine is a relatively specific inhibitor of transglutaminase and the function of transglutaminase is to introduce covalent cross-links. Therefore these papers provide arguments in favor of the transglutaminase hypothesis, whether the enzyme increases or decreases the frequency of microscopic inclusions. Karpuj *et al.* (102) did not observe a reduction of inclusions but Dedeoglu *et al.* (103) did. They suggested that their earlier beginning of cystamine treatment (at 2 weeks of age instead of 7) was responsible for better alleviation of the disease and clear reduction in size and number of inclusions (see ref 103, page 8946, Figure 4).

3.10. Presence of isodipeptide and transglutaminase 2 in the aggregates found in Huntington disease

Using immunostaining, transglutaminase 2 and its regulator calmodulin have been found in nearly all the inclusions in Huntington disease. It was suggested that calmodulin might provide the Ca^{2+} necessary for *in situ* transglutaminase activation (104).

Using liquid chromatography with electrochemical detection, the concentration of the free cross-linking isopeptide was found to be increased by about three fold (compared to normal controls) in the cerebrospinal fluid of patients with Huntington disease (105), and by about five fold in the cortex and caudate of patients (103). The free isodipeptide of either spinal fluid or brain tissue represents the final product of proteolysis of the cross-linked proteins. In these studies, increased levels of the isodipeptide could have been the consequence of increased neuronal death rather than its cause, since normal controls were used. It would be important in such experiments to compare Huntington disease with other causes of brain damage in which there is extensive neuronal loss. The neurons of the anterior horn of transgenic mice carrying a full-length androgen receptor with Q_{112} were found to contain isopeptide cross-links by immunostaining (106). Nearly complete colocalization of isopeptide cross-links with nuclear aggregates of huntingtin was observed in the frontal cortex of patients with Huntington disease (107). As the specificity of the monoclonal antibody used for detection has been questioned, this demonstration cannot be regarded as definitive (108).

As mentioned under “polar-zipper formation” treatment of protein aggregates with concentrated formic acid can be used to determine the nature of the bonds that stabilize the aggregate. It has been shown that synthetic peptides containing Q_{20}, which are insoluble in a solution of SDS, are completely dissolved in 90% formic acid. It was concluded that hydrogen bonded beta-sheets, which presumably cause insolubility of polyQ-containing peptides, are dissociated in 90% formic acid (71). Therefore, resistance of any polyQ-containing aggregate must be ascribed to stronger bonds, such as the covalent bonds formed by transglutaminase. This was confirmed by the fact that cornified envelopes, which are cross-linked by

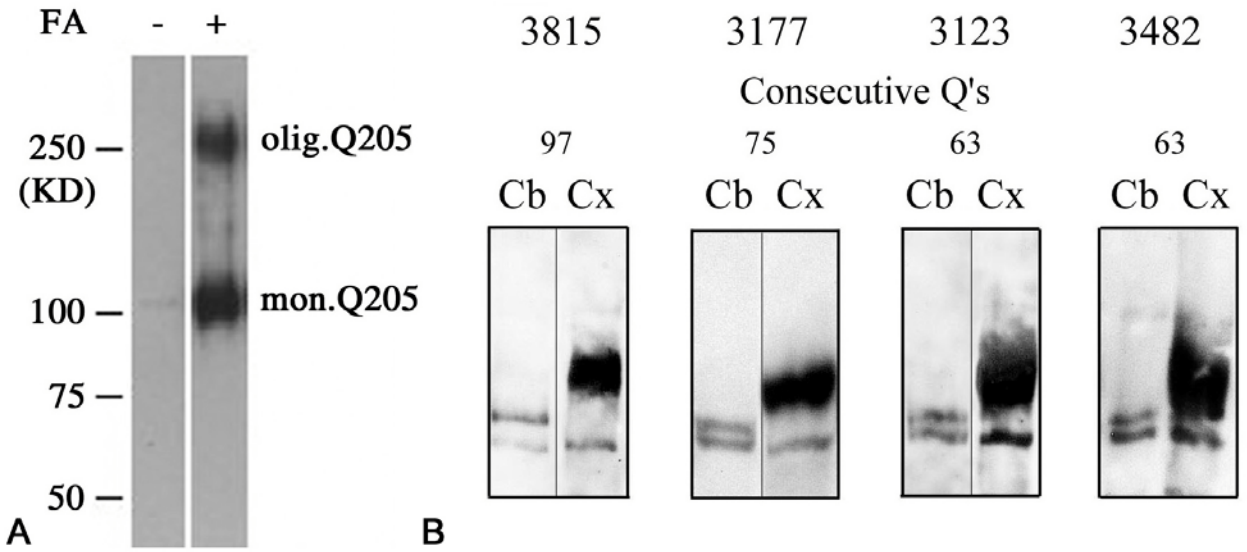

Figure 3. Oligomer containing an expanded polyglutamine formed in a cellular model and in cerebral cortex in Huntington disease. (A) Synthesis by PC12-Q205 cells of an oligomer containing expanded polyQ. Purified inclusions, with or without formic acid (FA) treatment, were boiled in SDS/2-ME and submitted to electrophoresis through 7,5% polyacrylamide. After transfer to nitrocellulose, the proteins were stained with the antibody 1C2, which recognizes specifically expanded polyglutamine. In the absence of formic acid pretreatment, neither monomer nor oligomer was detected. After pretreatment with 90% formic acid for 1 h at 37°C, electrophoresis revealed a strong band of monomer corresponding to an apparent molecular mass of ~100 kDa; the true molecular mass should be 36.340, but expanded polyQ reduces mobility in gel electrophoresis. In addition to the monomer, an oligomer with a considerably higher molecular mass is clearly stained by the 1C2 antibody. (B) Oligomeric state of soluble expanded huntingtin in cerebral cortex in Huntington's disease. Crude extracts of cortex and cerebellum were treated with 96% formic acid. Western blots prepared after electrophoresis were stained consecutively with anti-N-terminal antibody and 1C2. All specimens of cortex produced a broad oligomeric band of expanded huntingtin absent in the cerebellum. In contrast, all of the cerebellar specimens, but none of the cortical specimens, produced a clear band of expanded huntingtin. Reproduced from (71) with permission from PNAS.

the type 1 transglutaminase of keratinocytes (109) were resistant to formic acid. Incubation in concentrated formic acid of inclusions purified from cultured cells expressing an expanded polyQ released, in addition to the monomeric fragment containing the expanded polyQ, an expanded polyQ protein of size thought to correspond to a dimer (Figure 3). Work in our laboratory showed that the brain of patients with Huntington disease contained oligomeric and polymeric forms of expanded huntingtin, insoluble in formic acid. Of particular interest was the demonstration in cortical nuclei, of a formic acid-resistant polymeric aggregate that did not enter polyacrylamide gels and was too large to pass through a 0.2-micrometer filter (Figure 4). Neither the oligomer, nor the polymer was found in the cerebellum. Quantitative assay by infrared imaging gave a value for cortex 100 times greater than that of cerebellum. The resistance of oligomer and polymer to formic acid strongly suggested the participation of covalent bonds in their stabilization (71).

4. ALZHEIMER'S DISEASE

Alzheimer's disease is characterized by the deposition in the brain of extracellular senile plaque, the formation of intracellular neurofibrillary tangles and neuronal loss. Selkoe *et al.*, who were studying Alzheimer's disease, were the first to propose that transglutaminase could cause protein aggregation in neurological diseases (110). They proposed that transglutaminase stabilized the neurofibrillary tangles characteristic of Alzheimer's disease. This hypothesis was based on the fact that neurofilament proteins, which were then thought to be the main component of neurofibrillary tangles, could be cross-linked by transglutaminase *in vitro* (110). In further studies, Selkoe *et al.* found that paired helical filaments, which constitute the neurofibrillary tangles, were insoluble in 70% formic acid and therefore presumably stabilized by covalent bonds (111, 112). However it was later discovered that the main component of the neurofibrillary tangles was not neurofilament, but an abnormally phosphorylated form of the microtubule associated protein tau (113, 114). Tau has been shown to be an excellent substrate of tissue transglutaminase *in vitro* (115-117) and tissue transglutaminase has been found to colocalize with hyperphosphorylated tau in the neurofibrillary tangles (118).

The extracellular senile plaques were initially thought to be stabilized by non-covalent bonds because they were soluble in solution containing SDS (119, 120). However, it was later shown that senile plaques contained immunodetectable transglutaminase (121) and that the peptides or proteins predominantly composing the senile plaques (amyloid beta precursor protein, amyloid beta

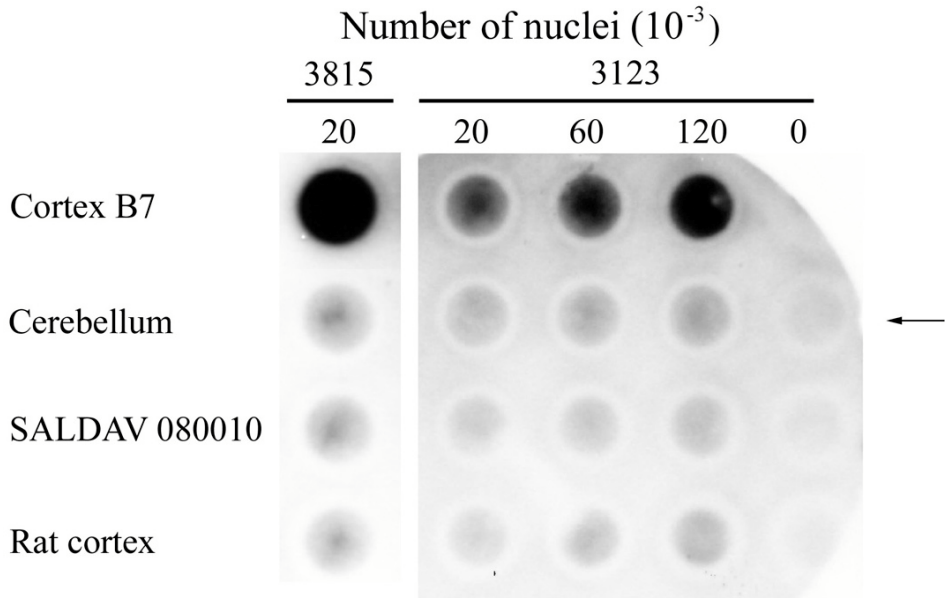

Figure 4. Polymeric state of expanded huntingtin in cerebral cortex in Huntington disease. Purified nuclei of cortex and cerebellum of brain 3815 were extracted with 96% formic acid. The suspension was diluted with a 10-fold excess of Tris/SDS/2-ME, boiled for 5 min, and immediately passed through cellulose acetate filters. The retained material was visualized with antibody 1C2. Control SALDAV 080010 was a lymphoblast line possessing a huntingtin with 79 glutamine residues. Rat cortex, whose huntingtin possesses Q8, should not be stainable by 1C2. Cortex 3815: 20,000 nuclei gave a strong signal. The corresponding cerebellum, SALDAV 080010, and rat cortex gave weak signals. Cortex 3123: 120,000 nuclei produced a weaker signal than 20,000 nuclei of cortex 3815, in keeping with a number of inclusions smaller in 3123 than in 3815. Arrow indicates control without nuclei. Resistance to formic acid of the cortical polymer of the two patients strongly suggests its stabilization by covalent bonds. Reproduced from (71) with permission from PNAS.

peptide and the non-amyloid beta component) were good substrates of transglutaminase *in vitro* (122-126).

4.1. Transglutaminase activity in Alzheimer's disease brain

Tissue transglutaminase activity of prefrontal cortex has been found to be increased by approximately three fold in Alzheimer's disease compared to age-matched controls, and to Alzheimer's disease cerebellum, a region which is usually spared in Alzheimer's disease and where neurofibrillary tangles are not detected (127). Another study described significant elevation of the level and activity of transglutaminase-1 and -2 in Alzheimer's disease brain, but this elevation was observed in both cortex and cerebellum (3). Its significance is therefore doubtful.

4.2. Presence of isodipeptide in Alzheimer's disease

Using an immunological method, the concentration of free isopeptide has been shown to be increased (~ 2 to 8.5 fold) in the cerebrospinal fluid of patients with Alzheimer's disease (128). Immunoblots and densitometric analysis of the staining showed a significant increase (45%) in the amount of cortical isopeptide in Alzheimer's disease, compared to normal control. Similarly, isopeptide crosslinks in tau are significantly increased in Alzheimer's disease (129). These crosslinks occur early in the disease (stage II) (130). Another study showed that the frequency of cross-links within the brain proteins insoluble in SDS/2-ME reaches up to 5-10% of total lysine residues and that cross-linked residues are increased by 30 to 50 fold in the Alzheimer's disease cortex and cerebellum compared to normal human brain (3). However, it must be pointed out that cross-linking of proteins by transglutaminase increasingly occurs during normal ageing in brain; this might explain why only quantitative differences between the normal brain of older persons and that of Alzheimer's disease were found (131). In all of the immunological studies of the isopeptide, the same monoclonal anti-isopeptide antibodies were used. We have already pointed out the doubtful specificities of these antibodies (108).

5. PARKINSON'S DISEASE

Parkinson's disease is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and by the aggregation of alpha-synuclein and other proteins in cytoplasmic aggregates called Lewy bodies. The involvement of transglutaminase in Parkinson's disease has also been suggested, but there are only a few studies on the subject.

Tissue transglutaminase was able to catalyze the formation of alpha-synuclein aggregates *in vitro* and in cultured cells. Treatment of cultured cells with cystamine significantly reduced the formation of alpha-synuclein aggregates (132). The substantia nigra of patients with

Parkinson's disease contained alpha-synuclein SDS resistant oligomers that could be immunoprecipitated with the anti-glu-lys isopeptide antibody (133). The level of cytoplasmic crosslinked alpha-synuclein monomer correlated with disease progression, whereas that of cytoplasmic monomeric alpha-synuclein was negatively correlated. Cross-linking of alpha-synuclein appears to be an early step in Parkinson's disease, preceding the aggregation of alpha-synuclein within the Lewy bodies. Transglutaminase might therefore be involved in the early stages of the disease. Others showed that the SDS-resistant oligomers of alpha-synuclein found in Parkinson's disease were in part recognized by the anti-isopeptide antibody (134). The halo of Lewy bodies was stained by antibodies directed against the isopeptide, transglutaminase and alpha-synuclein (132).

6. PERSPECTIVES

Many studies support the causative role of transglutaminase in the protein aggregation observed in a number of diseases of the central nervous system, particularly those caused by a polyQ expansion. However all these studies fall short of conclusive proof that transglutaminase action stabilizes the aggregates. What is lacking in order to prove unequivocally a role for transglutaminase is the demonstration of covalently cross-linked polymers containing isopeptide bridges. This could be accomplished by purifying the aggregates of affected brain and demonstrating the presence of isopeptide. Purification could be based on the insolubility of the aggregates in denaturing agents such as SDS and urea. These purified aggregates should then be terminally proteolyzed. This procedure would release free isopeptide, since the isopeptide is resistant to proteases. The most sensitive and specific methods for measuring the isopeptide would likely associate liquid chromatography and mass spectrometry (MS). The relative chromatographic retention time and the specificity of the fragments obtained by multiple MS should unambiguously identify the isopeptide. Indisputable evidence of cross-linking by isopeptide will provide firm support for therapeutic or prophylactic measures based on the role of transglutaminase.

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