A Neprilysin Polymorphism and Amyloid- β Plaques after Traumatic Brain Injury

Victoria E. Johnson,^{1–3} William Stewart,³ David I. Graham,³ Janice E. Stewart,³
Amy H. Praestgaard,⁴ and Douglas H. Smith^{1,2}

Abstract

Traumatic brain injury (TBI) induces the rapid formation of Alzheimer's disease (AD)-like amyloid- β (AB) plaques in about 30% of patients. However, the mechanisms behind this selective plaque formation are unclear. We investigated a potential association between amyloid deposition acutely after TBI and a genetic polymorphism of the AB-degrading enzyme, neprilysin ($n = 81$). We found that the length of the GT repeats in AB-accumulators was longer than in non-accumulators. Specifically, there was an increased risk of AB plaques for patients with more than 41 total repeats ($p < 0.0001$; OR: 10.1). In addition, the presence of 22 repeats in at least one allele was independently associated with plaque deposition ($p = 0.03$; OR: 5.2). In contrast, the presence of 20 GT repeats in one allele was independently associated with a reduced incidence of AB deposition ($p = 0.003$). These data suggest a genetically linked mechanism that determines which TBI patients will rapidly form AB plaques. Moreover, these findings provide a potential genetic screening test for individuals at high risk of TBI, such as participants in contact sports and military personnel.

Key words: axonal injury; human β -amyloid; neprilysin; polymorphism; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is increasingly implicated as a cause of progressive neurodegenerative disorders. In particular, TBI has emerged as the most important environmental risk factor for the development of Alzheimer's disease (AD) (Mortimer et al., 1985; Nemetz et al., 1999; Guo et al., 2000). The first pathological clue linking TBI with AD was the observation that amyloid- β (AB) plaques, a hallmark finding in AD, are found in up to 30% of humans who die acutely following TBI (Roberts et al., 1994). What is particularly surprising is the rapidity by which these plaques develop, appearing as early as 3 h post-trauma (Roberts et al., 1994).

This rapid plaque generation may be explained in part by extensive disruption of axonal transport after trauma. Specifically, axonal pathology, one of the most common and important pathologies of TBI, results in the massive accumulation of both amyloid precursor protein and its cleavage product, AB (Smith et al., 1999; Iwata et al., 2002; Smith et al., 2003; Chen et al., 2004; Uryu et al., 2007). It has been postu-

lated that lysis and breakdown of damaged axons may permit the expulsion of AB into the parenchyma for plaque formation (Chen et al., 2004). At a much slower pace, this general process has also been implicated as a mechanism of plaque formation in AD (Stokin et al., 2005).

It has been proposed that a polymorphism in the apolipoprotein E (ApoE) gene promote AB plaque formation after trauma in a subset of patients (Nicoll et al., 1995). However, there remains the opposing possibility that some TBI patients are better equipped to clear AB than others. Indeed, considering that AB production may be a common consequence of TBI (Smith et al., 2003; Chen et al., 2004; Uryu et al., 2007), endogenous amyloid-degrading enzymes such as neprilysin may play key roles in protecting against plaque pathology. If so, patients with a deficient capacity to degrade AB may be more prone to plaque formation acutely after TBI.

Neprilysin has emerged as the major AB-degrading enzyme *in vivo* (Iwata et al., 2000; Shirotani et al., 2001). In neprilysin knockout mice, endogenous AB has been found to accumulate in a gene-dose dependent manner (Iwata et al.,

¹Penn Center for Brain Injury and Repair, The University of Pennsylvania, Philadelphia, Pennsylvania.

²Department of Neurosurgery, and ⁴Biostatistics Analysis Center, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania.

³Academic Unit of Neuropathology, Division of Clinical Neuroscience, Institute of Neurological Sciences, Southern General Hospital NHS Trust, Glasgow, United Kingdom.

2001). In addition, neprilysin has become increasingly implicated in the amyloid pathology of AD (Yasojima et al., 2001; Iwata et al., 2005). Furthermore, we have recently found evidence that neprilysin plays a role in human TBI, with an increase in neprilysin immunoreactivity observed in association with AB accumulation for up to 3 years following trauma (Chen et al., 2009). Notably, this association in long-term survivors of TBI was typically found in the absence of AB plaque formation, suggesting a role of neprilysin in AB catabolism after injury.

Previously, a microsatellite polymorphism in the promoter region of the neprilysin gene has been examined in the contexts of AD and cerebral amyloid angiopathy (Sodeyama et al., 2001; Oda et al., 2002; Lilius et al., 2003; Yamada et al., 2003; Sakai et al., 2004; Wood et al., 2007). The location of this polymorphism in the promoter region suggests it may be important in modulating gene expression (Haouas et al., 1995; Ishimaru and Shipp, 1995) and thus may modulate the degree of AB degradation. Accordingly, in the present study we investigated the ability of a GT repeat polymorphism in the promoter region of the neprilysin gene to predict the development of AB plaques following TBI.

Methods

Case selection

A previously established cohort of patients was selected for study (Nicoll et al., 1995). Brain tissue from 90 patients (23 women and 67 men) in whom there was a history of acute, severe TBI was obtained at autopsy following diagnostic evaluation by the Department of Neuropathology, Southern General Hospital, Glasgow, U.K. All tissue was collected between 1987 and 1991, and approval for its inclusion in this study was provided by the appropriate research ethics committee.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Roberts et al., 1994). Briefly, at least two cortical areas were examined from each patient, including the entire temporal lobe and parietal cortex. All tissue was formalin-fixed and paraffin-embedded. Following pre-treatment with 80% formic acid, the sections were incubated overnight with a monoclonal antibody specific to amyloid- β (1/6F/3D) at a dilution of 1:1000. Processing was performed according to a previously defined protocol (Gentleman et al., 1989). Microscopic examination of slides was performed blind to clinical and demographic information, and the amount of amyloid- β deposits present rated using a modified version of the protocol of the Consortium to Establish a Registry of Alzheimer's Disease (CERAD) (Mirra et al., 1991). A positive case was defined as having one or more cortical areas containing a minimum number of deposits equivalent to the rating of "sparse" per the modified CERAD protocol (Roberts et al., 1994).

Genotyping

DNA extractions were performed in two distinct brain regions from all cases (the hippocampus and the cerebellum). Briefly, 10- μ m sections were cut from blocks of paraffin-embedded, formalin-fixed tissue from each case. Following

dewaxing using xylene and ethanol, proteinase K digestion was performed overnight at 56°C. The proteinase K was then inactivated by incubation for 10 min at 95°C.

The extracted DNA (two samples per case) was then amplified using the polymerase chain reaction (PCR). The primers for the PCR were TTTCAGTATGAATTCCGCACT (forward) and TGATCCCTTCTCTTTTGAAT (reverse). Reverse primers were tagged with Cy-5 dye (Eurogentec, San Diego, CA) to permit fragment sizing. PCR was performed using a hot start reaction with AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA). The reactions had a final volume of 15 μ L and contained 0.8 μ L of tissue DNA extraction product. PCR conditions started with initial denaturation at 95°C for 10 min, followed by 38 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C.

Fragment sizing analysis was performed using the Beckman Coulter CEQ™ 8000 Genetic analysis system (Fullerton, CA), with analysis completed by the accompanying software (version 8). All genotyping was performed blind to the amyloid status.

Statistical analysis

Statistical analysis was performed to determine an association between plaque formation and (1) the frequency of each individual allele, and (2) the total combined length of the GT repeat in both alleles. The Fisher's exact test was used to determine associations between all groups. Bonferroni-type adjustments were not necessary since repeated statistical testing was not performed. In addition, we were interested in understanding the relationship between the outcome and each independent variable studied. Odds ratios were generated using logistic regression. The Spearman's rank correlation was used to demonstrate correlations between total length of repeat and amyloid deposition. All statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC).

Results

Using an established cohort of head-injured cases ($n = 90$) (Nicoll et al., 1995), genotyping was possible in 81 cases. All 81 cases were European Caucasians aged from 0.15–79 years (mean 33.2 years). The survival times after TBI ranged from 4 h to 25 days (mean 3.45 days). Causes of injury included motor vehicle collisions ($n = 47$), falls ($n = 29$), and physical assault ($n = 5$). Post-mortem delays ranged from 5–120 h (mean 46.2 h) (Table 1). The groups were well matched for survival time and post-mortem delay.

Immunohistochemistry was previously characterized using the specific antibody 1/6F/3D (Roberts et al., 1994). Immunohistochemistry demonstrated that 19 of the total of 81 cases (23%) were positive for amyloid- β plaques within 25 days of having experienced a TBI (Table 1). Morphologically, the amyloid deposits were diffuse in nature, similar to those seen in early AD patients (Fig. 1). Based on the available clinical records, all AB-positive cases had no known clinical history of cognitive deficit, AD, or Down's syndrome prior to TBI.

Using fragment sizing to determine the length of a GT repeat polymorphism upstream of exon 1, five alleles were identified. Each allele represents a fragment containing 19, 20, 21, 22, or 23 GT repeats. These five alleles generate a potential

TABLE 1. CLINICAL CHARACTERISTICS OF THE CASES

	All cases (n = 81)	A β -positive (n = 19)	A β -negative (n = 62)
Age (y)			
Mean $\pm$ SD	33.22 $\pm$ 20.90	48.31 $\pm$ 20.08	28.60 $\pm$ 19.00
Range	0.15–79 y	14–75 y	0.15–79 y
Survival (d)			
Mean $\pm$ SD	3.45 $\pm$ 4.21	3.49 $\pm$ 4.27	3.44 $\pm$ 4.23
Range	4 h–25 d	15 h–11 d	4 h–25 d
Post-mortem delay (h)			
Mean $\pm$ SD	46.21 $\pm$ 30.37	48.26 $\pm$ 33.19	45.58 $\pm$ 29.71
Range	5–120 h	8–120 h	5–118 h

for 15 different genotypes of which 10 are represented in our total cohort. Analysis of two brain regions (the hippocampus and the cerebellum) was undertaken in each case. No differences were found in the GT repeat fragment size between these two regions in any cases, indicating an absence of regional genetic variation.

The presence (of at least one) of each of the alleles in both the amyloid-positive and amyloid-negative groups is shown in Table 2. The frequency of allele 22 was increased in cases positive for amyloid plaques ($p = 0.03$; Fisher's exact test). In contrast, the frequency of allele 20 was increased in the plaque-free group ($p = 0.003$).

In addition, the combined length of the repeat across both alleles was associated with the presence of plaques after injury. Specifically, the presence of a combined total GT repeat >41 was associated with an increased likelihood of having plaques ($p < 0.00005$) (Table 3). A positive correlation between total length of repeat and the presence of plaques following TBI was demonstrated with a correlation coefficient of 0.4 (95% CI: 0.11, 0.15; $p = 0.0005$; Spearman's rank correlation). The odds ratio (OR) for the development of amyloid plaques post-TBI in carriers of the 22 allele versus those

without this allele was 5.2 (95% CI: 1.2, 21.8; $p = 0.025$). The OR for the development of amyloid plaques post-TBI in carriers of a total GT repeat number >41 versus those with a total repeat number <41 was 10.1 (95% CI: 3.1, 32.5; $p = 0.0001$).

Additional analyses were also undertaken following the exclusion of all cases aged >60 years at the time of death to eliminate any bias from age-associated amyloid deposition. As previously demonstrated, no cases aged 60 or less were found to have amyloid deposits in non-TBI control cases (Roberts et al., 1994). All findings maintained significance within this subgroup with $p < 0.05$. Specifically, the frequency of allele 22 was increased in cases positive for amyloid plaques ($p = 0.03$), whereas 4 of 13 (31%) cases that were amyloid-positive had at least one allele 22 repeats long, as opposed to just 9 of 58 (16%) in the amyloid-negative group. The frequency of allele 20 was increased in the plaque-free group ($p = 0.016$), in which 7 of 13 (54%) cases that were amyloid-positive had at least one allele 20 repeats long, as opposed to 50 of 58 (86%) cases in the amyloid-negative group. Finally, the presence of a combined total GT repeat >41 was associated with an increased likelihood of TBI-associated plaques ($p = 0.0003$) in those under 60 years of age, while 9 of 13 (69%) cases that were amyloid-positive had repeat lengths >41 , compared to just 9 of 58 (16%) cases that were amyloid-negative.

Discussion

The results from this study demonstrate a strong relationship between a neprilysin polymorphism and amyloid- β plaque pathology shortly after TBI. Specifically, a promoter region polymorphism in the neprilysin gene predicted the presence of acute amyloid- β plaques following TBI. The length of this GT repeat polymorphism appears to be of critical importance, with post-TBI plaque deposition significantly more likely if an individual carries a longer repeat (>41 total GT repeats). This suggests that the specific GT repeat length in the promoter region influences the level of neprilysin available for A β clearance. In addition, two individual alleles were

FIG. 1. Diffuse amyloid- β plaques identified using specific antibody 1/6F/3D in the temporal lobe (fusiform gyrus) of a 30-year-old man. The plaques measure approximately 100 μ m in diameter. The patient died 16 h following severe TBI caused by motor vehicle accident (neprilysin genotype: 21/22).

TABLE 2. NUMBER OF CASES WITH AT LEAST ONE ALLELE PRESENT IN AMYLOID-POSITIVE AND AMYLOID-NEGATIVE CASES

GT repeat allele	Amyloid-positive (n = 19) % (no. cases)	Amyloid-negative (n = 62) % (no. cases)
19	5.2 (1)	16.1 (10)
20	52.6 (10)	87.1 (54)
21	63.2 (12)	50.0 (31)
22	26.3 (5)	6.5 (4)
23	10.5 (2)	4.8 (9)

identified with opposing associations with AB plaques: one associated with increased (allele 22), and one with decreased (allele 20) risk of AB plaques. These findings implicate neprilysin as having an important role in post-traumatic AB metabolism and plaque formation. These data may have important implications for individuals at high risk for TBI, such as participants in contact sports or military personnel. Specifically, a genetic screening test could potentially identify those at risk of developing AD-like AB plaques after TBI.

AB-plaque formation following TBI was initially described by Roberts and colleagues (Roberts et al., 1994), and has since been verified by other groups (Ikonovic et al., 2004), including our own (Smith et al., 2003; Uryu et al., 2007). These plaques, although more frequently diffuse in nature, are similar to those observed in early AD. However, whereas plaques in AD develop insidiously and almost exclusively in the elderly, TBI-induced plaque pathology can be found rapidly, within hours of injury and across the age spectrum (Roberts et al., 1994). The mechanisms of plaque formation post-trauma have not been fully elucidated. One possible mechanism may involve axonal pathology, a common pathology of brain injury (Adams et al., 1982; Geddes et al., 1997; Geddes et al., 2000). Impaired axonal transport following axonal injury is responsible for generating massive accumulations of both amyloid precursor protein and its cleavage product, AB, in disconnected axon terminals (Smith et al., 1999; Iwata et al., 2002; Smith et al., 2003; Smith et al., 2003; Chen et al., 2004; Uryu et al., 2007). Lysis and expulsion of the contents of these degenerating axons may provide an acute and copious source of AB for rapid plaque formation.

It remains curious, however, that not all patients with axonal pathology form plaques. Indeed, plaques are found in just 30% of all TBI cases and are comprised of a spectrum of TBI severities, age groups, and causes of injury (Roberts et al., 1994). The surprising lack of obvious clinical predictors raises suspicion that the propensity to develop post-traumatic plaques is genetically determined. In part, this was confirmed by the observation of an association between the ApoE ϵ 4 allele

and AB plaques following TBI, thought to be associated with an enhanced capacity to form AB (Nicoll et al., 1995). Here we show it is not simply the genesis of AB that is involved in causing plaque pathology, but that AB degradation and its genetic influences are mechanistically important. In essence, plaques may fail to develop in 70% of patients following trauma due to an individual's ability to efficiently clear rapid amyloid accumulation via neprilysin. The interaction between the neprilysin polymorphism reported here and the ApoE polymorphism will be important to determine. It is unclear whether these polymorphisms operate in a synergistic fashion to influence AB deposition. The numbers in this cohort are insufficient to appropriately investigate the role of both these polymorphisms simultaneously with respect to the presence of plaques, but this will be important future work if effective risk stratification is to be achieved.

Neprilysin, a membrane zinc-metalloendopeptidase, has emerged as the primary AB-degrading enzyme *in vivo* (Iwata et al., 2000; Shirotani et al., 2001), and is increasingly implicated in the pathogenesis of AD (for review see Iwata et al., 2005), with one study demonstrating that AD patients had a significant reduction in neprilysin mRNA in brain areas vulnerable to AB plaque deposition (Yasojima et al., 2001). Neprilysin is transcribed in a tissue-specific manner (Li et al., 1995; Turner et al., 2001), and is capable of degrading both monomeric and oligomeric forms of AB (Kanemitsu et al., 2003). Indeed, neprilysin knockout mice have been shown to accumulate AB (1–40) and AB (1–42) in a gene-dose-dependent manner (Iwata et al., 2001). We have shown that following TBI, immunoreactivity to neprilysin increases for at least up to 3 years following injury (Chen et al., 2009). This increase in neprilysin may be a response that is regulated by the genesis of AB itself in a positive feedback loop (Mohajeri et al., 2002; Pardossi-Piquard et al., 2005).

The precise mechanism by which this polymorphism affects AB plaque deposition following TBI is unknown. Notably, the location of the polymorphism in the promoter region has been previously suggested to effect neuronal transcription of the type one isoform of neprilysin (Ishimaru and Shipp, 1995). As such, it is conceivable that the specific GT repeat length in this region influences the level of neprilysin available for AB clearance. Interestingly, studies of the role of neprilysin in the psychiatric field suggest that shorter lengths of this polymorphism are associated with increased neprilysin expression (Comings et al., 1999). The mechanism by which a repeat with a difference of just a few base pairs might shift gene expression enough to have physiologically significant consequences is intriguing. It has been proposed that microsatellite polymorphisms, such as the noted GT repeat, can promote a conformational change within the DNA (Ishimaru and Shipp, 1995; Comings, 1998), which may be involved in the regulation of transcription (Comings, 1998).

While there appears to be an important role for this GT repeat polymorphism of neprilysin in TBI, it is less clear what role it may play in other disease states in which AB accumulation is also characteristic. Of four studies examining AD and this GT repeat, only one suggested an association. In addition, while this polymorphism was found to be associated with severity of cerebral amyloid angiopathy in another study, it was the shorter length of total repeats that was linked to increased amyloid accumulation in blood vessels. This is somewhat in contrast to our findings, in which AB pathology was

TABLE 3. TOTAL GT REPEAT LENGTH AND AMYLOID STATUS

Total GT repeat length	Amyloid-positive (n = 19) % (no. cases)	Amyloid-negative (n = 62) % (no. cases)
<41	36.8 (7)	85.5 (53)
>41	63.2 (12)	14.5 (9)

associated with longer total repeats. Thus it is difficult to interpret the differences between studies, which appear to highlight the complexity of *AB* metabolism in different disease states.

Our data implicate neprilysin as having an important role in *AB* clearance following TBI. The ability to identify patients at risk of forming *AB* plaques following TBI provides a unique opportunity for further study to determine how this affects both short-term clinical outcome, and more importantly, how this might contribute to the later development of AD. Ultimately, the identification of a genetically susceptible subgroup of patients who form *AB* plaques may provide the mechanistic link between TBI and AD. In addition, TBI is a major concern for certain segments of the population, particularly those involved in military action or contact sports. Our data may provide a means to identify individuals at risk of plaque development prior to TBI, and allow the development of an important screening test.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Douglas H. Smith, M.D.

Department of Neurosurgery

University of Pennsylvania

105 Hayden Hall

3320 Smith Walk

Philadelphia, PA 19104-6316

E-mail: smithdou@mail.med.upenn.edu