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ORIGINAL ARTICLE

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Real-time PCR quantitation of FE65 a β-amyloid precursor protein-binding protein after traumatic brain injury in rats

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Abstract In cases of traumatic brain injury (TBI) in which the patient survived for only a short period of time and was without macroscopic changes at autopsy, it is difficult to diagnose TBI. To detect early diagnostic markers of diffuse axonal injury (DAI), real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in an experimental head trauma model of rat was chosen. The β-amyloid precursor protein (β-APP) is a well-known diagnostic marker of DAI which can be detected by immunolabeling as early as 1.5 h after injury. β-APP has a binding protein, FE65, which is expressed in the brain of Alzheimer's disease patients along with β-APP, but no involvement with brain injury has been reported. Neuron-specific enolase (NSE) is also a useful marker of DAI. We found that FE65 expression increased dramatically as early as 30 min after injury and decreased after peaking 1 h post-injury, although NSE showed no significant changes. These results suggest that real-time PCR of FE65 mRNA is useful for the diagnosis of DAI in forensic cases.

Keywords Real-time PCR · Diffuse axonal injury · FE65 · β-APP · NSE

Introduction

In forensic autopsy cases with no signs of subdural hemorrhage, epidural hematoma, or brain contusion, and in which the patient survives only a short time following head injury, it is very difficult to diagnose brain injury. Recent studies in animal [1, 2, 3] and human brain tissues [4, 5, 6, 7, 8, 9, 10, 11] have shown that β-APP is a marker for diffuse axonal injury (DAI) in traumatic brain injury cases. β-APP is a cell-surface protein and a normal component of neuronal cells [4]. It is processed by the Golgi apparatus and carried along axons by fast anterograde transport [12, 13]. DAI has been reported after traffic accidents [14, 15, 16], falls [14, 17], and assaults [18, 19] and is produced by mechanical forces shearing the fibers at the moment of impact [20, 21, 22]. A large number of traumatic brain injury cases are fatal in less than 1 h and before DAI histology changes can be detected. Histology changes associated with DAI, such as axonal retraction balls or axonal enlargement, are difficult to detect with routine staining methods until 12–15 h post-injury [14, 23, 24, 25]. Many other techniques including immunohistochemical labeling have been developed to diagnose DAI in its early stages [4, 5, 6, 7, 9, 17, 26, 27], although these still require at least 1.5 h after injury to be able to detect DAI [28].

Recently, the β-APP adapter protein FE65 was identified [29, 30, 31], which is a binding protein expressed in brain tissues and nerve ganglia [29, 32, 33, 34, 35]. FE65 has been proposed as an important adapter protein of a multiprotein complex associated with the β-APP intracellular domain [29] and has been detected in the brain of Alzheimer's disease patients [34, 36, 37, 38, 39]. FE65 overexpression in cultured cells promotes β-APP translocation to the cell surface and increases β-amyloid peptide secretion [32]. It also regulates cell movement [40], but other functions are unclear [40]. To date there has been no attempt to identify FE65 involvement in traumatic brain injury. In the present study, FE65 mRNA was chosen to study DAI because FE65 directly binds to the β-APP intracellular domain and FE65 up-regulation may be related to β-APP accumulation in DAI. In addition, neuron-specific enolase (NSE), which has been detected as early as 1.5 h after injury by immunostaining [28], has also been shown to be a reliable marker for the diagnosis of DAI [28, 41]. NSE is localized in neurons and axons [42, 43] and NSE mRNA is expressed in multiple neuronal regions

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[44]. NSE is an important glycolysis enzyme, converting 2-phospho-D-glycerate to phosphoenolpyruvate [45]. In the field of brain injury pathology NSE has been reported as a diagnostic marker for DAI [28], cerebral ischemia [46, 47] and status epilepticus [44]. The NSE detected by immunohistochemistry is concentrated by interruption of axonal transport [13, 48, 49, 50, 51, 52, 53].

Since a method for specific mRNA quantitation by the polymerase chain reaction (PCR) has been developed [54, 55], the real-time reverse transcriptase-polymerase chain reaction (RT-PCR) method has been used in many fields, including neuropathology [56, 57, 58], demonstrating its applicability for studying brain injury pathology [59]. This study evaluated the efficiency of the real-time PCR method for FE65 and NSE mRNA quantitation and early DAI diagnosis in rats.

Materials and methods

Experimental head injury

Sprague-Dawley (SD, *n*=38) male rats, weighing between 550–650 g, were divided into 9 groups: naive controls (*n*=3), sham operations (*n*=2), and 0.5, 1, 3, 6, 12, 24, and 48 h (*n*=4–5 each) post-traumatic brain injury groups. Animals were kept under a 12 -h light-dark cycle with food and water ad libitum. Traumatic brain injury was induced using a slightly modified version of the experimental head trauma model of Marmarou et al. [60]. Animals were anesthetized with sodium pentobarbital (50 mg/kg intraperitonally). Instead of the 10 mm diameter metal disk used in the original model, a coin (Japanese 10-yen coin, diameter 23.5 mm, width 1.5 mm, weight 4.5 g, 95% copper) was cemented to the skull vault with one drop of dental cement at the midline area of the coronal and lambdoid sutures. With this modification, depression skull fractures can be avoided. Since the coin diameter is bigger than the transverse diameter of the rat skull (less than 20 mm), the coin edge does not touch the skull. In addition, heavier rats were employed to prevent skull fracture. After the rat was placed on a foam bed, a 500 g copper weight was raised and allowed to fall freely from 1.5 m through a Plexiglas tube onto the coin. Sham-operated animals received identical anesthesia and surgery, but not the impact injury.

The experimental procedures followed the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and were in accordance with the Guidelines for Animal Experimentation for our university.

Tissue preparation

At 0.5, 1, 3, 6, 12, 24, and 48 h (*n*=4–5 each) after traumatic brain injury, rats were sacrificed with a lethal dose of pentobarbital. The brain was removed without perfusion, then cut into six coronal sections. One section, which included the right cerebral cortex, subcortical white matter, corpus callosum, and hippocampus, was immediately frozen at –80°C until used for RNA extraction. A second section was fixed in 10% paraformaldehyde in 0.1 M sodium phosphate buffer and paraffin-embedded for histopathological preparation.

Total RNA preparation

Tissue was excised from one frozen section, containing the right cerebral cortex, subcortical white matter, corpus callosum, and hippocampus. The opposite side of the brain section was kept frozen in case of technical failure. The tissue was immediately homogenized. Total RNA was prepared from the homogenate using the PolyATtract System 1000 (Promega, Madison, WI) following the manufacturer's protocol.

Primer and probe design for real-time PCR

FE65 mRNA was amplified with the primers FE65 mRNA forward primer (5'-GCCTCCTTCTGCTGTCACATG-3') and FE65 mRNA reverse primer (5'-AGGCTGTGCAGGCTGCA-3') which produced an 89 bp PCR product. NSE mRNA was amplified with the primers NSE mRNA forward primer (5'-TGATGACCTGAC-GGTGACCA-3') and NSE mRNA reverse primer (5'-CAAACA-GTTGCAGGCCTTCTC-3') which produced a 91 bp PCR product. Detection probes (TaqMan Probe, PE Applied Biosystems, Foster City, CA) specific to rattus FE65 mRNA and NSE mRNA were as follows: FE65 mRNA probe 5'-(FAM)TGCGAGCCCAATGCTG-CCAGT(TAMRA)-3' and NSE mRNA probe 5'-(FAM)CCCCA-AGCGCATCGAGCGG(TAMRA)-3'. Primers and TaqMan probes were designed using the primer design software Primer Express 1.5 (PE Applied Biosystems) based on the common rat cDNA sequence region for the establishment of a quantitative assay method for gene expression levels using real-time PCR. We used primers and a VIC-labeled rodent TaqMan probe for detection of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (PE Applied Biosystems).

Real-time PCR

Real-time PCR is a method for measuring the accumulation of PCR products during the reaction with a spectrophotometer. RT-PCR, which amplifies cDNA, is an indispensable method for quantifying small amounts of RNA. Real-time PCR templates were obtained by a reverse transcriptase reaction of total RNA using AmpliTaq Gold DNA polymerase (PE Applied Biosystems). The template was mixed with Platinum Quantitative PCR SuperMix-UDG, which contains 1.5 U Platinum Taq DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, and 1 U uracil DNA glycosylase (Invitrogen, Carlsbad, CA), TaqMan probe, and 0.05 µM of each primer. All reactions were performed in an ABI Prism 7700 sequence detector (PE Applied Biosystems). The thermal cycling conditions included a 95°C predenaturation step for 10 min, thermal cycling with 45 cycles of 95°C denaturation for 15 s and 60°C annealing and extension for 1 min. The amount of cDNA in the samples was estimated with standard curves representing the log of the input amount (log starting cDNA molecules) as *x* and the threshold cycle as *y*. The expression rate was obtained by normalizing the amount of FE65 mRNA or NSE mRNA to that of GAPDH.

Histopathological procedure

For histopathological studies, brains were sectioned in the coronal plane, before paraffin-embedding. Brain histopathological changes were observed in the coronal sections at the frontal horn level. Sections (5 µm thick) were cut with a rotary microtome and prepared with Bodian staining to verify the axonal changes.

Results

Histopathology

Experimental head trauma resulted in diffuse axonal damage in rat brains as determined by Bodian staining and the observed axonal changes are shown in Table 1. In control animals, axons were manifested in a longitudinal or transverse arrangement as thin fibers (Fig. 1A). In the groups sacrificed at 0.5 and 1 h after injury, only a few axons

Table 1 Axonal changes observed using Bodian staining

Groups	Axonal changes		
	Waving	Enlargement	RB
Control (*n*=3)	– – –	– – –	– – –
Sham (*n*=2)	– –	– –	– –
0.5 h (*n*=4)	– – – +	– + – –	– – – –
1 h (*n*=4)	+ – – –	– + – –	– – – –
3 h (*n*=5)	+ + + – +	– – – – –	– – – – –
6 h (*n*=5)	+ + + + –	– – – + +	– – – – –
12 h (*n*=5)	+ + + + +	+ + + + +	+ + – + –
24 h (*n*=5)	+ + + + –	+ + + + –	+ – – + –
48 h (*n*=5)	+ + + + +	+ – – – +	– – – + –

Each symbol indicates the result for 1 rat.
(+) or (–) indicates the presence or absence of waving, of axon, axonal enlargement or RB, respectively.
RB, retraction balls.

showed waving and enlargement (Fig. 1B). In the 6-h group, the numbers of enlarged and waving axons increased and retraction balls were seen in the 12-h group (Fig. 1C). The alterations were most severe in rat brains 24 h after injury (Fig. 1D).

Real-time PCR

Before analyzing samples by real-time PCR, the presence of both FE65 and NSE mRNA in each sample was confirmed by agarose gel electrophoresis. Figure 2A shows the time course of FE65 mRNA expression after brain injury. The significant induction of FE65 mRNA expression was observed as early as 0.5 h following injury, reaching a peak at 1 h ($p<0.05$), and gradually decreasing to the control level 12 h after injury. Interestingly, it increased again at 24 h, finally decreasing at 48 h.

Figure 2B shows the time course of NSE mRNA expression after traumatic brain injury. Although the NSE gene expression showed a peak at 24 h after injury, there were no significant changes in NSE mRNA expression when normalized to GAPDH expression.

Discussion

In previous studies, many approaches have been carried out for establishing the forensic pathological diagnosis of traumatic brain injury. Several markers or changes in human brain tissue after traumatic brain injury have been investigated using immunohistochemistry [28, 61, 62, 63, 64, 65, 66, 67, 68, 69]. The samples used in these studies were derived from the brains of autopsy cases that showed obvious macroscopic changes such as skull fracture, epidural hemorrhage, subdural hemorrhage, subarachnoidal hemorrhage or cerebral contusions. DAI can be found in cases that sustained mild traumatic brain injury with no macroscopic changes at autopsy. It is difficult to diagnose DAI in patients who survived for only a short time after injury because DAI cannot be detected until 12–15 h post-injury with routine staining methods [14, 23, 24, 25]. The aim of the present study was to discover an early sensitive marker of DAI. In previous studies, the principal method for diagnosing DAI was immunohistochemistry, and β-APP and NSE were detectable in human brain as early as 1.5 h post-injury [28]. The axonal damage induces a local inter-

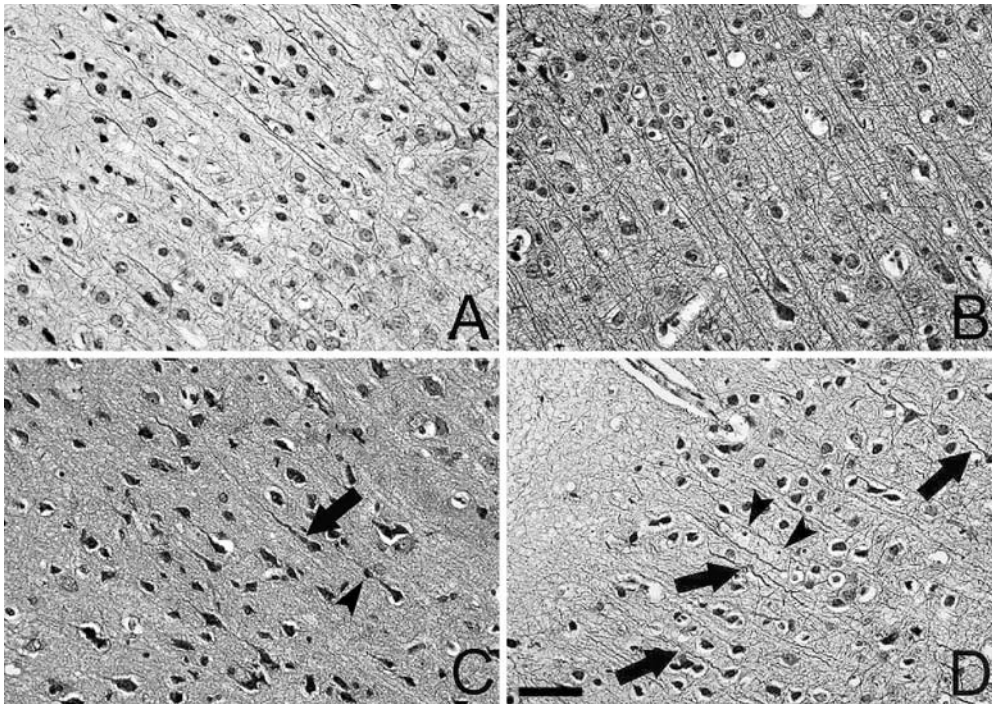

Fig. 1 **A** Bodian staining in the parasagital subcortical white matter of the control rat, **B** 1 h injured rat, **C** 12 h injured rat and **D** 24 h injured rat. Waving and enlargement of axons (*arrows)* and retraction balls (*arrowheads*) were observed in the rat that survived greater than 6 h. The findings were severe in the 12 h and 24 h injured rats (**C, D**). (*Bar* 100 μm)

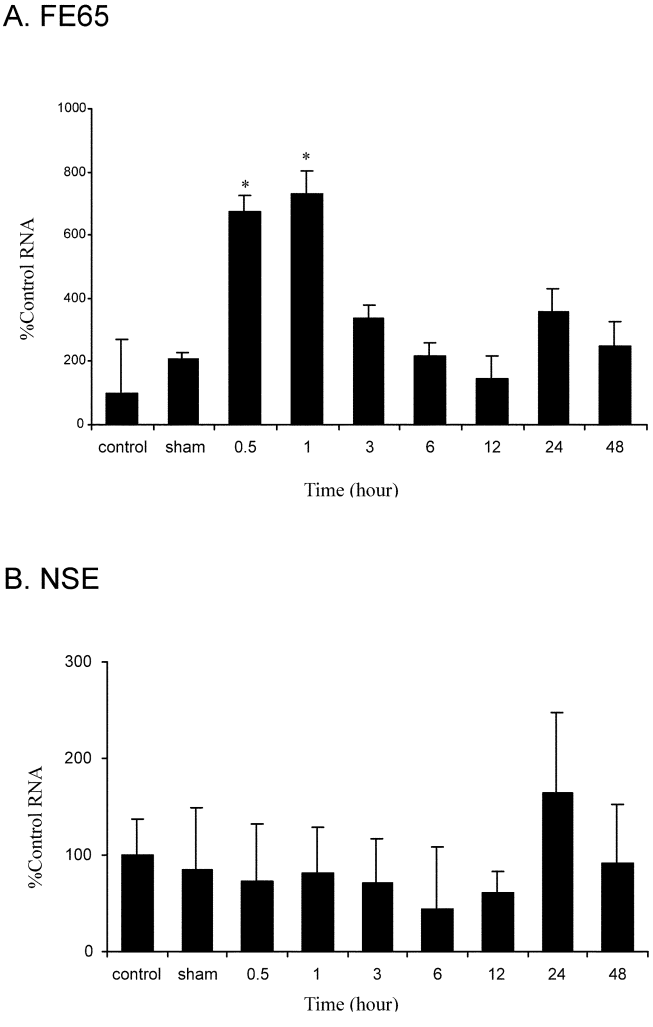

Fig. 2 **A** Real-time quantitative PCR analysis of FE6**5** and **B** NSE mRNA expression in the rat brain. mRNA levels are expressed as a percentage of control animal expression. One-way ANOVA was performed, followed by the Dunnett's multiple comparison test to evaluate statistical significance (*p<0.05)

ruption of axonal transport, which makes both β-APP and NSE detectable. In experimental brain injury models, the detection of several different markers has been reported from between 1 h to 1 day after injury [70, 71, 72, 73, 74, 75]. Although tumor necrosis factor-α (TNFα) reactions were detected as early as 30 min after injury by immunoelectron microscopy [70], it may not be specific to DAI.

In the present study, the application of a recently developed real-time PCR method was established for the detection of FE65 and NSE mRNA. In comparison with other molecular biological methods, real-time PCR is a rapid and sensitive method for quantifying small amounts of PCR products [54] and RT-PCR products [55]. It allows one to analyze a relatively large number of samples in a short period of time [56]. Real-time PCR has many advantages, but also some limitations, including the requirement of expensive instrumentation [56]. The applicability and possible usefulness of quantitative RT-PCR have been shown in human forensic autopsy cases [76] and pathological autopsy cases [37]. Using cloned plasmid DNA as a standard and a housekeeping gene (GAPDH) to normalize the data, we were able to determine the absolute starting copy number of a given mRNA using real-time PCR. It should be emphasized that this quantitation is based on using plasmid DNA as the template for the standard measure of reverse-transcribed mRNA, with the assumption that the mRNA amplification rates are equivalent [58].

To investigate whether an early sensitive marker exists, we began with an animal model of traumatic brain injury. We chose the impact acceleration model [60] from several different experimental models available [60, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86] because it is economical, technically simple, and produces diffuse axonal injury similar to that described in humans without focal damage [87]. Our results showed widespread axonal damage, such as enlarged and waving axons in rats more than 6 h after injury and retraction balls, a hallmark of DAI, were seen after 12 and 24 h (Fig. 1) without obvious traumatic damage. These results are consistent with the histology of DAI described in humans [14, 23, 24, 25].

In this study, the FE65 mRNA expression level showed an increase at 0.5 h, reached a peak 1 h after injury and then decreased to control levels at 12 h. It increased again at 24 h, then returned to control levels at 48 h. On the other hand, NSE mRNA expression remained unchanged up to at least 48 h after injury.

Our study found that the real-time PCR method is a sensitive procedure for detecting FE65 mRNA in the rat brain in the early stages of DAI, as early as 30 min post-injury. Of all the proteins that localize in axons, FE65 may be implicated in DAI and reacts to the damage at a very early stage. Although the results of real-time PCR for NSE are inconsistent with previous immunohistochemical studies [28, 41], there is a similar report by Schreiber et al. who reported that the NSE mRNA level decreased 5 days after injury [44]. NSE may react to damage later than FE65.

In conclusion, using real-time PCR we have demonstrated that FE65 mRNA is an early DAI diagnostic marker in the experimental impact acceleration rat model. Our aims for future studies are to apply this detection method to human autopsy cases in forensic practice and to investigate how FE65 protein is involved in the pathogenic mechanism of DAI formation, since our method demonstrates several advantages over previous studies. FE65 is a sensitive and specific marker of DAI and is induced as early as 30 min after injury. Real-time PCR is a rapid and sensitive method for quantifying small amounts of RT-PCR products [55] in a short time. Our quantitative method may be better than morphological analysis for diagnosing DAI in forensic practice [70]; evaluating the samples morphologically is often difficult because of changes in the postmortem period. To the best of our knowledge, this is the first report employing real-time PCR for the study of DAI. These results are expected to support the diagnosis of DAI in forensic cases in which the patient survived for only a short time after traumatic brain injury and were without obvious focal damage.

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