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ORIGINAL ARTICLE

Two different immunostaining patterns of beta-amyloid precursor protein (APP) may distinguish traumatic from nontraumatic axonal injury

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Abstract Immunostaining for beta-amyloid precursor protein (APP) is recognized as an effective tool for detecting traumatic axonal injury, but it also detects axonal injury due to ischemic or other metabolic causes. Previously, we reported two different patterns of APP staining: labeled axons oriented along with white matter bundles (pattern 1) and labeled axons scattered irregularly (pattern 2) (Hayashi et al. (Leg Med (Tokyo) 11:S171-173, 2009). In this study, we investigated whether these two patterns are consistent with patterns of trauma and hypoxic brain damage, respectively. Sections of the corpus callosum from 44 cases of blunt head injury and equivalent control tissue were immunostained for APP. APP was detected in injured axons such as axonal bulbs and varicose axons in 24 of the 44 cases of head injuries that also survived for three or more hours after injury. In 21 of the 24 APP-positive cases, pattern 1 alone was observed in 14 cases, pattern 2 alone was not observed in any cases, and both patterns 1 and 2 were detected in 7 cases. APP-labeled injured axons were detected in 3 of the 44 control cases, all of which were pattern 2. These results suggest that pattern 1 indicates traumatic axonal injury, while pattern 2 results from hypoxic insult. These patterns may be useful to differentiate between traumatic and nontraumatic axonal injuries.

Keywords Traumatic axonal injury · Beta-amyloid precursor protein (APP) · Hypoxia · Immunostaining patterns

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Introduction

Beta-amyloid precursor protein (APP) is a transmembrane glycoprotein synthesized in neurons [1]. While the actual physiological role of APP is unclear, it may have roles in cell/cell and cell/matrix interactions, growth promotion, and autocrine functions, as well as acting as a binding site for various substances, including heparin [2]. APP is transported along axons to the synapse by fast anterograde transport and is undetectable in normal axons by immunohistochemistry. However, brain insult induces an increase of APP transcription and interrupts its transport, resulting in localized accumulation of APP to detectable levels in the form of axonal bulbs. The detection of APP-positive axonal bulbs is an effective tool for the diagnosis of diffuse traumatic axonal injury in forensic practice [2–9]. The detection of axonal bulbs using conventional hematoxylin and eosin (H-E) or silver staining requires a survival time of at least 15–18 h following head injury. However, immunostaining for APP can detect axonal bulbs as early as 2–3 h after head injury [2–9]. Moreover, some axonal bulbs can be detected at 35 min [10] or 90 min [11] after head injury depending on the case.

Although diffuse axonal injury has been considered the consequence of head trauma, several investigators have emphasized that axonal bulbs can form in the absence of head injury. These axonal bulbs form usually in the presence of both intracranial and systemic pathology, such as cerebral infarction/hemorrhage, meningitis, bronchopneumonia, hypoglycemia, and drug intoxication as well as in cases of ventilator support [5, 7, 12–15]. In particular, hypoxia and ischemic brain damage without head injury show the formation of axonal bulbs. Posttraumatic edema and cerebral and cerebellar herniation secondary to head injury may also cause axonal bulbs. Therefore, it is necessary to distinguish the detection of APP after traumatic axonal injury from other axonal injuries [7, 12–15].

Previously, we reported a preliminary study that suggested that two different immunostaining patterns of APP may distinguish traumatic from nontraumatic axonal injuries [16]. However, no APP-positive staining was observed in control cases with hypoxia/ischemia in the absence of head injury, and therefore, the pattern of hypoxic axonal injury could not be elucidated in that study. Accordingly, in the present study, we performed further investigation using a larger number of head injury cases and control cases and discuss whether the two different immunostaining patterns depict trauma and hypoxic/ischemic brain damage, respectively.

Materials and methods

Forty-four cases of blunt head injury (age range, 2 to 93 years; mean age, 61.0 years; 34 males and 10 females; range of survival time after injury, short time (<10 min) to 90 days) and the same number of control cases showing hypoxia/brain ischemia without head injury (age range, 2 to 92 years; mean age, 54.9 years; 25 males and 19 females) were collected from the Department of Legal Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University. The details of the head injury cases and control cases are summarized in Tables 1 and 2, respectively. From each case, formalin-fixed, paraffin-embedded sections of corpus callosum were immunostained for APP as described previously [11, 16]. Briefly, after deparaffinization and heating to 95 °C in citrate buffer (10 mM, pH 6.0) for 20 min, the sections were immersed in 0.3 % H_2O_2-phosphate-buffered saline (PBS; pH 7.2) for 30 min to block endogenous peroxidase activity. The sections were then rinsed with PBS and incubated in PBS containing 1 % normal goat serum and 1 % bovine serum albumin (BSA) to reduce nonspecific reactions and with mouse anti-APP monoclonal antibody (clone 22C11, Millipore, Darmstadt, Germany; 1:50) at 4 °C for 14 h. Thereafter, the sections were rinsed three times for 5 min in PBS and incubated with biotinylated goat anti-mouse IgG (1:100) at 18 °C for 1 h. After rinsing in PBS, the sections were incubated with LSAB2 (labeled streptavidin-biotin; DakoCytomation) at 18 °C for 30 min, and positive signals were visualized using 0.02 % 3, 3′-diaminobenzidine. The sections were dehydrated and mounted without counterstaining.

Semiquantitation of APP-positive axonal bulbs was performed under ×200 magnification using a light microscope (field area calculated as 0.64 mm^2). All sections were examined independently and "blind" to the original diagnosis by two of the authors (T. H. and M. O.). Axonal bulbs were counted as an average from ten fields in each case. According to our previous study [16], the stainings of labeled injured axons, such as varicose axons (sinusoidally swollen axons) and waving axons, were classified into two patterns: labeled axons oriented along with white matter bundles (pattern 1) and axons scattered irregularly (pattern 2). All sections were then reexamined by both observers to reach a consensus.

Results

In 24 of the 44 cases of head injury that survived for ≥3 h, at least one axonal bulb per ×200 microscopic field (0.64 mm^2) was labeled by APP (Fig. 1 and Table 1). Injured axons, including varicose and waving axons, were also labeled by APP in head injury case, and usually associated with APP-positive axonal bulbs (Fig. 1). Similar to the results of our previous study [16], the staining patterns of labeled injured axons could be divided into two types in 21 of the 24 APP-positive cases (Fig. 1). As shown in Table 1, pattern 1 alone was observed in 14 cases, pattern 2 alone was not observed, and both patterns were detected in 7 cases. In the cases with both patterns, more than five APP-labeled axonal bulbs per ×200 microscopic field (0.64 mm^2) were observed and the survival time after injury ranged from 1 to 14 days. In the remaining 3 of the 24 cases, clear patterns were not determined because, in those cases, there were no labeled varicose and/or waving axons which were needed for classification.

APP-labeled injured axons were also detected in 3 of the 44 control cases (Fig. 2). In those cases, causes of death were sepsis, myocardial infarction, and delayed asphyxia due to ligature ligation, respectively, with survival times from 2 to 14 days (Table 2). In all three cases, the staining patterns of labeled axons were pattern 2 alone (Fig. 2).

Discussion

Several studies have indicated different types of APP immunostaining between traumatic and hypoxic axonal injuries. Graham et al. [8] suggested multifocal and "Z"-shaped patterns, while linear and geographical patterns were described recently by Davceva et al. [17]. These patterns of APP immunostaining indicated the outline of an infarct and, therefore, resulted from vascular complications of an elevated intracranial pressure. Oehmichen et al. [18] suggested that a wave-like pattern may be produced in response to impulsive head rotation or other type of mechanical impact, while the irregularly aggregated pattern may result from hypoxic insult. In the cases reported here, the Z- shaped pattern was not observed. Our results are appropriate since no histological findings of secondary infarction were observed in any cases. In the wave-like pattern, the injured axons were scattered but confined to individual white matter bundles, so that

Table 1 Details of the cases with head injury

Case no.	Age range and gender	Survival time	Cause of head injury	Brain lesions	APP[a]	Pattern
1	80s M	90 d	Falling	SDH	++	[b]
2	90s F	24 d	Assault	SDH	+	1
3	80s M	21 d	Traffic accident	SDH, CC, ICH	−	
4	80s M	18 d	Assault	SDH, SAH, CC	−	
5	80s M	14 d	Assault	SDH, SAH	++	1, 2
6	60s M	12 d	Falling	SDH, SAH	++	1
7	40s M	9 d	Assault	EDH, SDH, SAH, CC	++	1, 2
8	30s M	7 d	Falling	SDH, SAH, CC	+	1
9	60s M	7 d	Falling	SAH, CC	+	[b]
10	60s M	7 d	Falling	SDH, SAH	−	
11	30s F	6 d	Assault	SDH, SAH	−	
12	60s F	5 d	Assault	SDH, SAH, CC	++	1
13	60s M	4 d	Assault	SDH	++	1, 2
14	2 M	3 d	Assault	SDH, SAH	++	1, 2
15	60s M	3 d	Falling	SDH, CC	−	
16	50s M	2.5 d	Falling	SDH	+	[b]
17	40s M	2.5 d	Assault	SAH	−	
18	70s M	2.5 d	Assault	SDH, SAH, CC	++	1, 2
19	20s M	32 h	Assault	SAH, CC	−	
20	40s M	1 d	Falling	SDH, CC	+	1
21	30s M	1 d	Assault	SDH, SAH, CC	++	1, 2
22	50s M	1 d	Assault	SDH, SAH, CC	++	1, 2
23	50s M	14 h	Falling	EDH, SDH, SAH	+	1
24	30s M	13 h	Falling	SDH, SAH	−	
25	50s M	12 h	Falling	SDH	+	1
26	50s M	9 h	Assault	SAH, CC	+	1
27	60s M	8 h	Falling	SDH, SAH, CC	−	
28	70s M	8 h	Falling	SDH, SAH, CC	+	1
29	60s M	7 h	Falling	SDH, SAH	++	1
30	60s M	7 h	Assault	SDH, SAH, CC	−	
31	80s F	6 h	Traffic accident	EDH, SDH, SAH, CC	+	1
32	80s M	6 h	Traffic accident	SAH	+	1
33	70s M	4.5 h	Traffic accident	SDH, SAH, CC	−	
34	50s M	4 h	Falling	SDH, SAH, CC	+	1
35	90s M	3 h	Falling	SDH, SAH, CC	+	1
36	80s F	2.5 h	Falling	SAH	−	
37	50s M	2 h	Falling	SAH, CC	−	
38	70s F	1 h	Falling	SDH, SAH, CC	−	
39	80s F	1 h	Falling	SDH	−	
40	60s F	0.5 h	Assault	SAH	−	
41	70s F	Short	Assault	SAH, CC	−	
42	20s M	Short	Assault	SAH	−	
43	70s M	Short	Falling	SAH	−	
44	40s F	Short	Assault	SDH	−	

APP beta-amyloid precursor protein, *EDH* epidural hematoma, *SDH* subdural hematoma, *SAH* subarachnoidal hemorrhage, *CC* cerebral contusion, *ICH* intracerebral hemorrhage

[a] −, 0 axonal bulbs (AB); +, 1–5 AB; ++, >5 AB per ×200 microscopic field

[b] Clear patterns were not detected

Table 2 Details of the control cases with no head injury

Case no.	Age range and gender	Survival time	Cause of death	APP[a]	Pattern
1	60s F	14 d	Sepsis	++	2
2	20s M	9 d	Methamphetamine intoxication	−	
3	50s M	8 d	Upper cervical spinal cord injury	−	
4	50s M	4 d	Myocardial infarction	+	2
5	80s F	4 d	Pulmonary fat embolism	−	
6	70s M	3 d	Bronchopneumonia	−	
7	80s F	2 d	Delayed asphyxia due to ligature strangulation	++	2
8	30s F	2 d	Liver failure due to liver cirrhosis	−	
9	70s F	1 d	Myocardial infarction	−	
10	9 F	1 d	Influenza encephalopathy	−	
11	70s F	7 h	Traumatic bilateral pneumothorax	−	
12	70s M	5 h	Myocardial infarction	−	
13	60s M	4 h	Traumatic shock due to chest bruise	−	
14	80s F	3 h	Myocardial infarction	−	
15	80s M	3 h	Exsanguination due to stab wounds	−	
16	80s M	2.5 h	Myocardial infarction	−	
17	2 F	2.5 h	Hyperthermia	−	
18	60s M	2 h	Organophosphorus intoxication	−	
19	40s M	1 h	Myocardial infarction	−	
20	40s M	25 m	Asphyxia due to food aspiration in the airway	−	
21	80s F	10 m	Asphyxia due to food aspiration in the airway	−	
22	80s M	Short	Myocardial infarction	−	
23	70s M	Short	Myocardial infarction	−	
24	40s M	Short	Pulmonary thromboembolism	−	
25	70s M	Short	Intrinsic subarachnoid hemorrhage	−	
26	50s F	Short	Hypertensive cerebral hemorrhage	−	
27	30s F	Short	Drowning	−	
28	50s M	Short	Asphyxia due to ligature strangulation	−	
29	30s M	Short	Asphyxia due to ligature strangulation	−	
30	20s F	Short	Asphyxia due to manual strangulation	−	
31	90s F	Short	Asphyxia due to smothering	−	
32	50s M	Short	Exsanguination due to stab wounds	−	
33	30s F	Short	Exsanguination due to stab wounds	−	
34	40s F	Short	Exsanguination due to stab wounds	−	
35	30s F	Short	Exsanguination due to stab wounds	−	
36	30s M	Short	Exsanguination due to incised wounds	−	
37	40s F	Short	Carbon monoxide intoxication	−	
38	30s M	Short	Hydrogen sulfide intoxication	−	
39	60s M	Short	Hydrogen sulfide intoxication	−	
40	10s M	Short	Butane intoxication	−	
41	30s F	Short	Alcohol intoxication	−	
42	80s M	Short	Chloroform intoxication	−	
44	70s M	Short	Organophosphorus intoxication	−	
44	50s M	Short	Burn shock	−	

APP beta-amyloid precursor protein

[a] −, 0 axonal bulbs (AB); +, 1–5 AB; ++, >5 AB per ×200 microscopic field

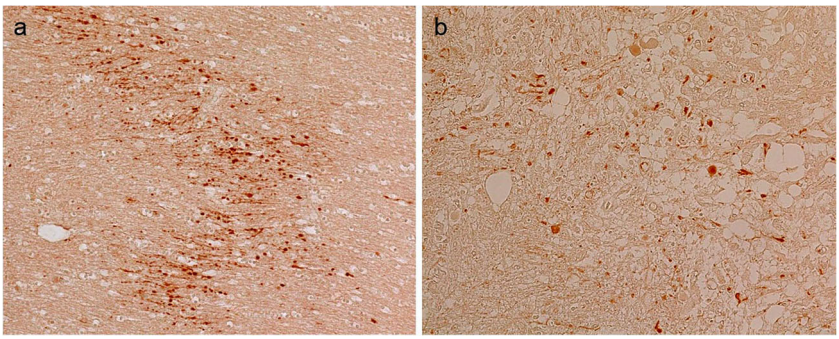

Fig. 1 Immunostaining for APP in a head injury case with 5-day survival (case 12 in Table 1). **a** Representative image of pattern 1 showing that APP-labeled axons oriented along with white matter bundles. **b** Representative image of pattern 2 showing that APP-labeled axons scattered irregularly in white matter. Original magnification, ×200

pattern 1 in our study may correspond to the wave-like pattern. In addition, the irregularly aggregated pattern resembles pattern 2 observed in our study. However, Oehmichen et al. [18] did not demonstrate the irregularly aggregated pattern in fatal hypoxic brain without mechanical impact [18].

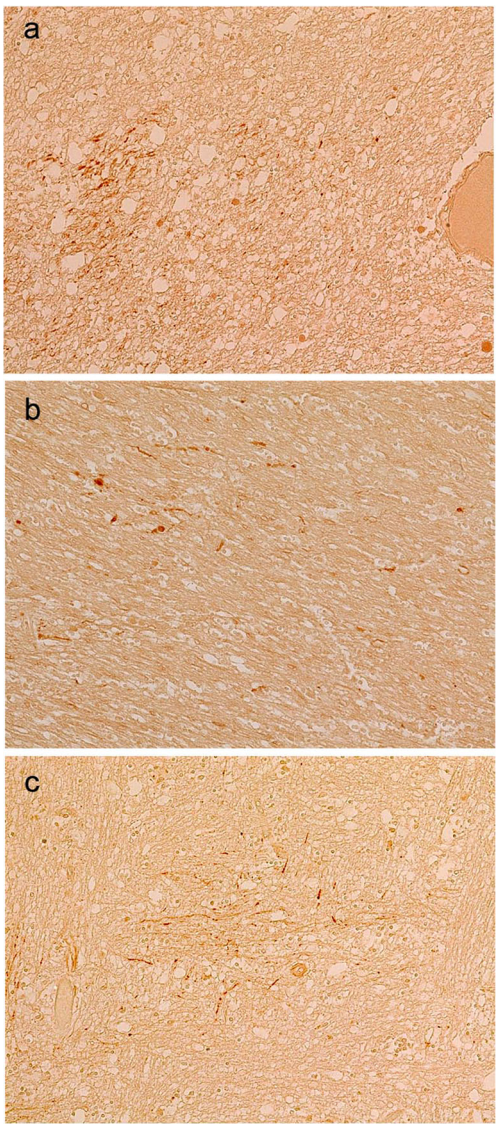

Fig. 2 APP-positive control cases (**a** case 1; **c** case 4; **b** case 7 in Table 2). In all cases, APP-labeled axons are scattered irregularly in white matter, indicating pattern 2. Original magnification, ×200

In the present study, APP-labeled injured axons were detected in 3 of the 44 control cases with hypoxia/brain ischemia in the absence of head injury. Harrington et al. [2] demonstrated that 11 of 20 cases with histological changes of hypoxia without head injury showed positive APP staining and survival times from 1 h to 3 months. Kaur et al. [14] reported that 12 of 25 cases of hypoxia without head injury showed positive APP staining and survival times from a few hours to 68 days. However, specific staining patterns of the labeled axons were not mentioned in their investigations [2, 14]. In our study, pattern 2 (irregularly scattered pattern) alone was detected in the three APP-positive control cases, in which more than one APP-labeled axonal bulb per ×200 microscopic field was detected. Moreover, pattern 1 (oriented along with white matter bundle pattern) alone was detected in 14 of the 44 head injury cases, while cases of pattern 2 alone were not observed. These results suggest that pattern 1 indicates traumatic axonal injury, and pattern 2 results from hypoxic insult. In head injury cases showing both patterns (cases 5, 7, 13, 14, 18, 21, and 22 in Table 1), pattern 2 axons may have resulted from hypoxia, ischemic brain damage, or edema complicated with the head injury.

Local cytoskeletal disruption due to shearing injury may be the initial and main cause of traumatic axonal injuries [14]. Therefore, it seems likely that the injured axons were confined along with the white matter bundles observed in the first pattern of our study, which corresponds to the direction of mechanical impact. On the other hand, a variety of factors, such as metabolic disturbances, loss of cellular membrane integrity, and ultrastructural changes as well as stretching effect due to edema, may associate with hypoxic axonal injuries [2]. Thus, it appears that the injured axons were scattered irregularly depending on a point of weakness to those factors, as observed in pattern 2 of our study.

In clinical and forensic fields, traumatic axonal injury is very difficult to diagnose because the brain appears quite normal on conventional computed tomography (CT) and magnetic resonance imaging (MRI) when the survival period is short.

Recently, advances in neuroimaging and laboratory techniques have allowed for more subtle lesions to be detected earlier. Diffusion tensor imaging (DTI) has shown some advantages for detecting axonal injury by acquiring water diffusion in different directions to provide microstructural information about axons or myelin [19–22]. This imaging technique can detect axonal injury within 24 h after head trauma, which was unremarkable in conventional MRI imaging [23, 24]. Moreover, Li et al. [25] demonstrated significant decrease of DTI parameters, including fraction anisotropy and axial diffusivity, corresponding to the axonal damage from 3 h after head trauma in a rat model. DTI is applicable to post mortem imaging in forensic practice, although facilities are limited at present. Additionally, our results suggest that it may be possible to differentiate between traumatic and nontraumatic axonal injuries by the patterns of decrease of DTI parameters.

In conclusion, our results suggest that two different patterns of APP staining in the corpus callosum may distinguish traumatic from nontraumatic injured axons. Several previous studies described differences in the anatomical distribution (corpus callosum, lentiform and caudate nuclei, thalamus, subthalamic regions, medulla, and pons) of APP-positive axons between traumatic and nontraumatic cases [9, 14, 15]. Accordingly, our results have a distinct advantage in enabling the differentiation using only one sample, corpus callosum. Further investigations are required to confirm whether similar results are observed in other anatomical sites of the brain.

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Conflict of interest The authors have no conflict of interest to declare.

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