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Is traumatic axonal injury (AI) associated with an early microglial activation? Application of a double-labeling technique for simultaneous detection of microglia and AI

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Abstract The aim of the present study was to determine whether axonal injury (AI) induces a microglial reaction within 15 days after brain trauma. In 40 selected cases of confirmed AI, the topographical relation of AI and microglial reaction was assessed using an immunohistochemical double-labeling technique for simultaneous demonstration of AI using β-amyloid precursor protein (β-APP) antibody and of microglia using CD68 antibody. Although traumatic injury was usually followed by a moderate early diffuse rise in the number of CD68-reactive cells in the white matter, increases in macrophages in areas of AI accumulation were only sporadic and did not occur until after 4 days. At survival intervals of 5–15 days a moderate microglial reaction in regions of β-APP-positive injured axons was detected, at maximum, in half of the case material. During this interval AI-associated satellitosis-like clusters or stars described by other authors after a survival time of more than 7 weeks were an isolated phenomenon. The prolonged microglial reaction as well as the reduction of β-APP-positive AI during longer survival periods supports the hypothesis that AI is not primarily chemotactically attractive and that the damage to a portion of β-APP-stained axons may be partly reversible. Most cases clearly require a prolonged interval of more than 15 days before initiation of the final scavenger reaction. For forensic purposes the increase in the number of microglial cells within the region of AI accumulation after a survival time of more than 5 days and the multiple and distinct demonstration of star-like microglial reactions within the white matter after survival times exceeding 7 weeks may provide valuable postmortem information on the timing of a traumatic event.

Key words Trauma · Axonal injury · Microglia · Immunohistochemical double labeling

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Introduction

The focal accumulation of β-amyloid precursor protein (β-APP) in axons is characteristic of sites of axonal injury (AI) and impaired fast axonal transport [4, 12, 18]. The phenomenon of AI is not specific [25, 27] and is observed, among others, following cerebral ischemia [9, 16] and in other types of white matter pathology [30]. AI is, however, especially common and severe in cases of mechanical head trauma with traumatic brain injury (TBI) at the moment of injury related to shear and tensile strains [13, 15].

It has long been known that a diffuse microglial reaction follows TBI [14, 17]. The occurrence of microglial clusters (see also [8]) in association with AI was described by McLellan et al. [19] and Adams et al. [1]. More detailed descriptions of this phenomenon were given by Povlishock and Becker [28], and by Geddes [9] and Geddes et al. [10], who noted the presence of microglial aggregates in the white matter after head trauma in addition to numerous injured axons. However, this was only consistently observed after survival times exceeding 7 weeks [1, 10, 28].

Reports of an association of AI with microglia have so far only referred to an increase in both AI and the number of microglia in the white matter of traumatically damaged brains. However, since microglial reactions [22] like microglial clusters [2] are nonspecific, the question arises whether a topographical – and thus functional – relation already exists between the two phenomena at an earlier time. This is especially important since the number of microglia is known to increase within a survival time of 24–72 h following TBI [17, 21]. We used an immunohistochemical double-labeling technique for simultaneous demonstration of AI (β-APP epitope [30]) and microglia (PG-M1 epitope of CD68 [3]) to obtain a reliable topographical classification of these two phenomena.

Materials and methods

Materials

Paraffin-embedded brain tissue from 40 individuals who had survived TBI for more than 3 h and up to 15 days was investigated. The selection included only cases already found to have a large number of β-APP-positive injured axons in a prior study [26]. Table 1 lists all 40 cases according to survival time and morphological diagnosis. The individuals ranged in age from 1 to 73 years; cases of both sexes were included.

Brain regions with numerous β-APP-positive fibers and bulbs, in particular the corpus callosum and the rostral segment of the pons, were examined. The investigation did not consider whether primary and/or secondary bleeding, edema, or ischemia were also present.

Methods

CD68 demonstrates the epitope PG-M1, which is specific for macrophages and microglia [6, 9]. Early AI was demonstrated using antibodies against β-APP, which is present in increased levels in hypertrophic fibers and/or bulbs due to axonal transport following AI [11, 30].

Immunohistochemical double labeling was performed as follows: after dewaxing of paraffin sections (about 5 μm thick) and blocking of the endogenous peroxidase with 7.5% H_2O_2, microwave pretreatment in citrate buffer (pH 6.0) was performed. The specimens were incubated with normal rabbit serum (Dako, Hamburg, Germany), diluted 1 : 5 with TRIS buffer (pH 7.6) for 30 min, followed by incubation with the first primary antibody (monoclonal mouse anti-β-APP, Boehringer Mannheim, Mannheim, Germany; diluted 1 : 50 with TRIS buffer) at room-temperature for 3 h. The secondary antibody (biotinylated rabbit-anti-mouse, Dako) was diluted 1 : 300 with TRIS buffer. The sections were incubated for 30 min in this solution, followed by a 30-min incubation period with horseradish peroxidase (HRP)-labeled avidin-biotin-complex (Dako). Visualization of the HRP was done with diaminobenzidine. After washing in distilled water, the specimens were subjected to enzyme pretreatment with pronase (0.01%, Merck, Darmstadt, Germany). They were then incubated with the second primary antibody (monoclonal mouse anti-CD68, Dako; 1 : 100 with TRIS buffer) at 8 °C for 18 h. Subsequent incubations with the secondary antibody (see above) and with the HRP-labeled avidin-biotin-complex were performed for 30 min. The sections were then treated for 15 min with biotinylated tyramine [20] at an effective dilution of 1 : 250 and incubated with an alkaline phosphatase (AP)-labeled avidin-biotin-complex (Dako, Hamburg) for 30 min. Visualization of the AP was done with new-fuchsin. Between each step the specimens were rinsed three times with TRIS buffer. Counterstaining was done according to the method of Mayer. The β-APP immunoreactive precipitate was brown coloured, the CD68-immunoreactive precipitate red.

Nonspecific staining was excluded by using serial sections of each paraffin block. The slides were alternately stained for the CD68-antigen and β-APP-antigen, using the above described protocol with the same antibodies, dilutions and reagents. Staining for β-APP was performed using microwave pretreatment. For staining with CD68 antibody, a combination of microwave and enzyme pretreatment was used. When compared with the serial sections, the immunohistochemical double labeling stained the same structures without alteration of immunoreactive precipitate intensity.

In Fig. 1 the results of the double-labeling technique are demonstrated: red indicates activated microglia (macrophages = CD68) and yellow-brown indicates injured axons (β-APP) on the same slide. The fine processes of the microglia cells did not express CD68 antigen using the dobule labeling method.

Table 1 Differentiation of the cases according survival time and final neuropathological diagnosis (*Cort. hem.* cortical hemorrhage, *Subd.* subdural)

Survival time	Cort. hem.	Subd. hematoma	Cort. hem. + Subd. hematoma	Total
< 24 h	1	1	3	5
25–48 h	4	1	3	8
49–72 h	2	2	3	7
73–96 h	3	3	2	8
5–10 days	6			6
11–15 days	4	2		6
Total	20	9	11	40

Results

A moderate diffuse increase in the number of CD68-positive cells was already visible in the apparently intact white matter of the brain after a post-traumatic interval of 24–96 h; there was, however, no remarkable association with AI. CD68-positive cells were most numerous in areas of hemorrhage, edema and/or cell destruction (Fig. 1 a). Between the 5th and 15th day of survival a moderate increase of microglial cells in the areas of AI-positive white matter (Fig. 1 b) could be demonstrated in half of all cases, while from the 1st to the 4th day little microglial reaction was seen in the areas with massive AI.

No direct topographical association was found between single microglial cells and β-APP-positive bulbs and/or fibers (Fig. 1 c, d). The microglial cells were generally confined to the neuropil and were located far from β-APP-positive axons and bulbs. Only in one individual case did microglia aggregate around β-APP-positive bulbs, simulating a satellitosis (Fig. 1 e). This case also exhibited phagocytosed β-APP-positive fragments in the cytoplasm of macrophages, obviously following secondary ischemia and edema (Fig. 1 f).

Discussion

The present study shows that microglial reaction as a general phenomenon is associated with TBI and can be detected in the white matter after post-TBI survival times of between 5 and 15 days. Up to the 4th day areas with β-APP-positive AI appeared to be spared from a microglial reaction.

A direct local bulb-related increase in the number of microglial cells, including the formation of satellitosis-like microglial clusters or stars, was detectable only in one case within the first 15 days of survival. This observation is in accordance with the findings of Povlishock and Becker [28], Adams et al. [1] and Geddes et al. [10], who detected microglial clusters only after survival times of at least 7 weeks.

The absence of a microglial reaction can be attributed to primarily deficient chemotactic attractivity and the lack of opsonizating amounts of IgG and/or complement on the surface of the damaged axons. As a consequence, the

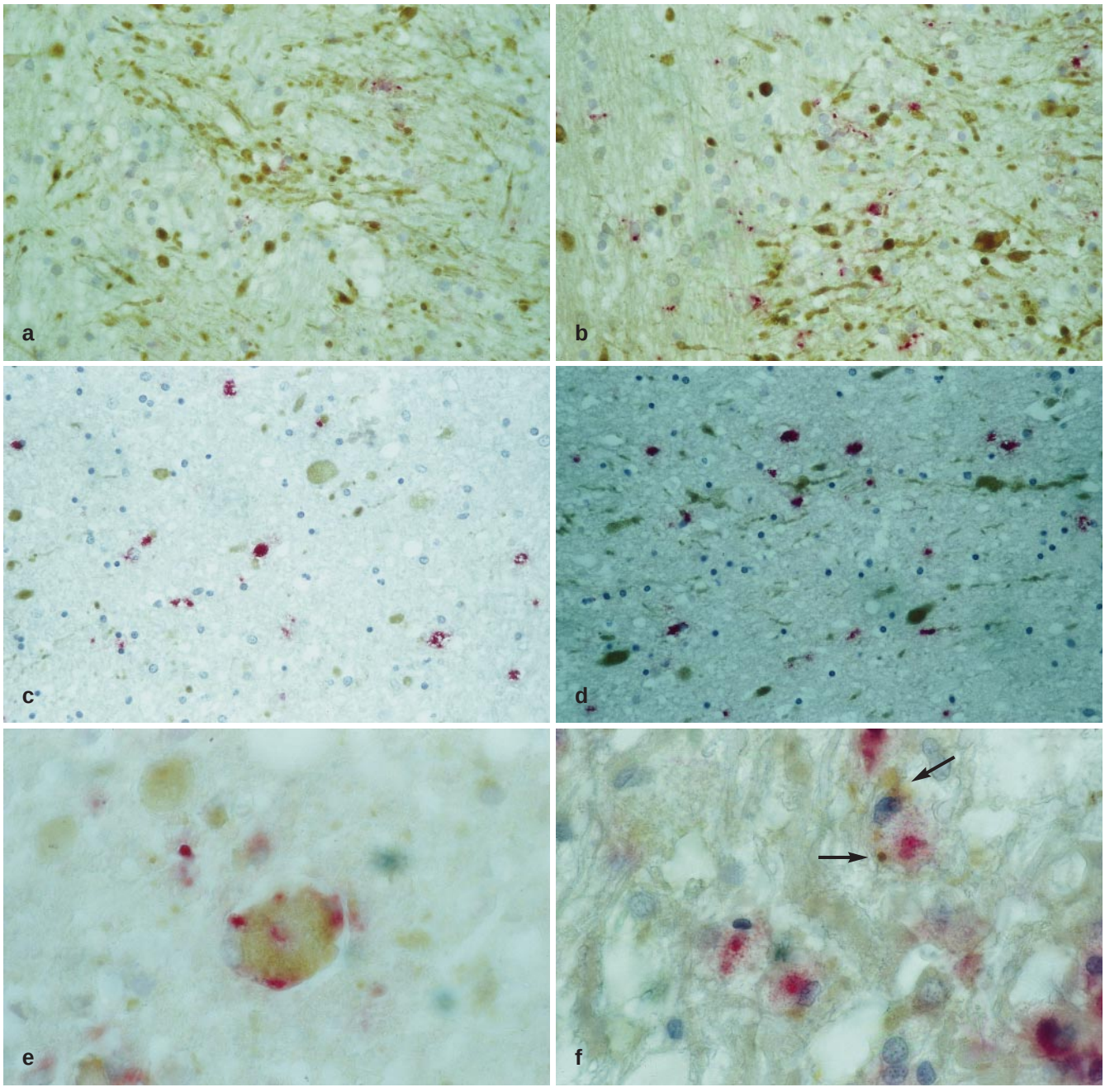

Fig. 1 a–g Double labeling for simultaneous detection of microglia (CD68 antibody, *red*) and AI (β-amyloid precursor protein, *brown*). **a** AI without microglial reaction (survival time 4 days); **b** diffuse, moderate microglial reaction in the region with AI demonstration (survival time 8 days); **c**, **d** isolated injured axons and diffuse increase in the number of microglia without local association with single injured axons (survival time 10 days); **e** injured axon surrounded by microglia cells as an indication of an incipient satellitotic process in a case with secondary hypoxic damage (survival time 14 days); **f** phagocytosis of β-APP-positive fragments by microglia (*AI* axonal injury). **a–d** × 100; **e** × 1000; **f** × 500

macrophages did not recognize the β-APP-positive axons in this phase as "foreign" or "necrotic", and therefore not as "damaged" and to be scavenged. The phagocytic process, which was first demonstrated by electron microscopy [28], could be confirmed by light microscopy within the 15th post-traumatic day in one case, but only in association with a secondary ischemic process.

During the early survival time it is possible that AI is a reversible phenomenon, i.e., that the positive β-APP reaction of the axons represents only a transient structural and biochemical change [7, 27, 29]. The observation of Geddes et al. [10] that individual bulbs may lose their reactivity to β-APP antibodies may be an additional explanation of the same phenomenon.

For forensic purposes it is important to achieve a temporal classification of the individual phenomena to enable an estimation of the age of the traumatic injury on post-

mortem tissue [23, 24]. Based on the present findings, an increase in the number of microglial cells in areas of AI can be observed after 5 days at the earliest. Individual microglial clusters in connection with AI did not regularly appear within a survival period of 15 days, but were obvious after survival times of more than 6 weeks. Moreover, if it is assumed that β-APP-positive AI appears approximately 3 h after TBI (cf. [26]), the simultaneous demonstration of microglia and AI should provide additional information on the temporal course following TBI.

Using the CD68 antibody only macrophages in the sense of activated microglia were demonstrated. Therefore, an activation of resting microglial which are reactive to HLA-DR or CD45 could not be excluded.

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