HISTOPATHOLOGY OF TRAUMATIC BRAIN INJURY TO THE DEVELOPING BRAIN

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ABSTRACT

Trauma to the developing brain leads to necrotic lesion at the site of injury and delayed apoptotic neurodegeneration at distant sites mainly in the thalamus, caudate nucleus and cortex. We studied the distribution and timely activation of monocytes/macrophages, microglia, astrocytes and two inflammatory cytokines, interleukin (IL)-1ß and IL-18, 2 h to 14 days following trauma using biochemistry and immunohistochemistry. A marked increase of mRNA and protein levels for IL-1ß and IL-18 was detected 2-12 h after injury. Apoptotic cell death affects mostly neuronal populations ipsilateral to the injury 6 h to 5 days later. Microglial activation was first evident at 12 h, peaked at 36-48 h and decreased substantially by 5 days. Astrocytic activation started at 18 h, peaked at 48 h and gradually declined by 14 days after trauma. The activation of immune and glial cells together with increased expression of both interleukins occurred at the site of primary and secondary damages. Our findings suggest that reactive microglia/astrocytes at the sites of secondary lesions might maintain apoptotic neurodegeneration over several days after traumatic injury to the immature brain but they might also promote tissue repair. Understanding the role of glial cells to progression of inflammation and apoptotic neurodegeneration together with tissue repair in the developing brain may provide valuable information to guide treatment after brain trauma.

Key words: *developing brain, trauma, apoptosis, microglia, astroglia*

INTRODUCTION

Brain trauma is one of the leading causes of morbidity and mortality among children. Brain damage provokes both a primary mechanical impact and secondary degenerative responses (2,11) that result in delayed cognitive impairments (13). Many previous reports have described the time course and extent of post-traumatic neurodegeneration after traumatic brain injury (TBI) (3,7,14). Macrophage accumulation, microglia activation and reactive astrogliosis are well known to occur following brain trauma (1,16,17). Recent experimental and clinical data indicate that apoptosis, inflammation and oxidative stress play a major role in the pathogenesis of neuronal death following TBI. Neuroinflammation represents a potential pathogenetic factor in many central nervous system (CNS) disorders including brain trauma (8). Microglia and astrocytes are involved in inflammatory events after brain injury (4), however their controversial roles in developmental TBI still remain to be elucidated. Clinical and experimental evidence suggests that the immature brain is unique in its response and vulnerability to TBI compared to the adult brain (12). Previously, we demonstrated that experimental trauma to the infant rodent brain

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leads to acute excitotoxic cell death in the area of impact and a delayed massive apoptosis in many brain regions (5). We reported also glial activation and expression of IL-1ß and IL-18 inflammatory cytokines after trauma to the immature brain (6). The present study investigates the timely and spatial interaction of these events using mainly single and double immunohistochemistry.

MATERIAL AND METHODS

Seven-day-old Wistar rat pups (BgVV, Berlin, Germany) were subjected to weight drop TBI or sham surgery as previously described (5). For RT-PCR and Western blotting, animals were sacrificed at 2, 6, 12, 24, 48, 72 h, 7 and 14 days later (n=4-5 per time point). Fresh tissue was taken from cortex, striatum and thalamus, snap frozen in liquid nitrogen and stored at -80°C for analysis. Total cellular RNA was isolated from snap frozen tissue by acidic phenol/chloroform extraction and DNAse I treatment (Roche Diagnostics), RNA was reverse transcribed and the resulting cDNA was amplified by PCR. Protein extracts were obtained from homogenized snap-frozen tissue, then centrifuged, denaturated and electrotransferred onto a nitrocellulose membrane. It was incubated with goat polyclonal anti-IL-1ß antibody (AB) (Santa Cruz Biotechnology) or goat polyclonal anti-IL-18 AB (R&D Systems).

For immunohistochemistry, animals survived 6, 12, 18, 24, 36, 48 h or 5, 14, 21 days (n=5 per time point). They were perfused with 0.01M PBS followed by 4% paraformaldehyde in 0.1M PB. The whole brains were removed, post-fixed for 3 days and embedded in paraffin. Coronal sections, 6-10 µm thick, were cut and processed for standard immunoperoxidase labelling. Mouse monoclonal ED1 AB (Serotec) was used to visualize blood-born macrophages and reactive macroglia. For detection of apoptosis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) using ApopTag® Peroxidase In Situ Apoptosis Detection kit (Oncor Appligene) was applied. For evaluation of microglia, Griffonia simplicifolia isolectin-B_4 (GSA I-B_4) (Sigma) was used. For demonstration of mature astrocytes, glial fibrillary acidic protein (GFAP) was detected using a rabbit polyclonal anti-GFAP AB (Chemicon International). Double staining for TUNEL and GSA I-B_4, TUNEL and GFAP or GSA I-B4 and GFAP was performed in representative sections. To detect IL-1ß and IL-18 containing cells, goat polyclonal anti-IL-1ß and anti-IL-18 ABs (Santa Cruz Biotechnology) were applied.

RESULTS

After mechanical trauma to the infant rat brain an immediate primary necrotic lesion was registered at the site of injury, i. e., the right parietal cortex and a subsequent apoptotic neurodegeneration at distant sites such as striatum, thalamus, various cortical regions and white matter tracts starting at 6 h later. The affected areas were largely infiltrated by ED1 immunopositive cells (Fig. 1). Both apoptotic degeneration and ED1(+) cell infiltration were further significantly increased in those areas at 12 h, and in addition spread to hippocampal dentate gyrus and subiculum. Cingulate, frontal, retrosplenial and parietal cortices, thalamic nuclei, caudate, dentate gyrus and subiculum were most severely affected. TUNEL and ED1 stainings were more expressed ipsilaterally to trauma but also in the contralateral site with a peak at 24 h and significant reduction by 48 h. At day 5, there was no significant difference with the controls that contained only single positive cells.

Trauma induced a strong activation of both microglia and astrocytes within cortical contusion area and at distant sites, severely affected by apoptotic neurodegeneration. For GSA I-B_4 labeled microglia, this activation started at 12 h after trauma, peaked by 36-48 h and decreased significantly by day 5. Following head trauma, increased numbers of amoeboid/transitional microglia mainly within white matter tracts and highly ramified microglia throughout the gray matter were found.

In control animals, GFAP(+) astrocytes were usually present mostly in white matter tracts, within the molecular cortical layer and in the border zones between gray and white matter. Initially a significant increase in the densities of GFAP immunoreactive astrocytes (hyperplasia) and an obvious increase in intensity of staining (hypertrophy) were first detected within corpus callosum and cortex by 18h after trauma. Maximal expression of GFAP(+) cells were found at 48 h after trauma. Astrocytic activation was observed in the regions of apoptotic

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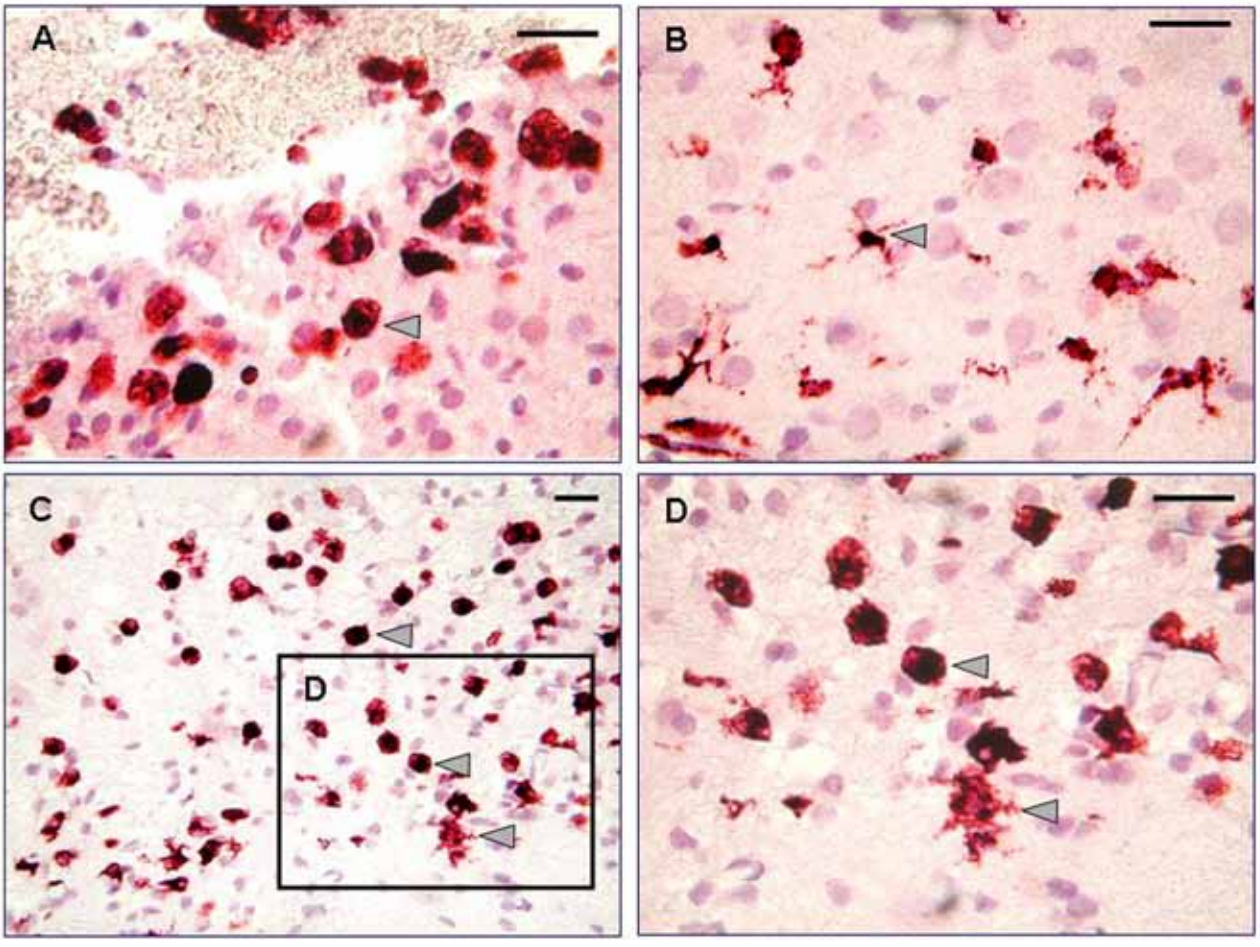

Fig. 1. ED1(+) cells in the right parietal cortex (A), thalamus (B) and capsula interna (C, D) 24 hrs after trauma to 7-day-old rat brain, VNR substrate and hematoxylin counterstaining, bar =25 μm

cell death, including white matter tracts. It decreased significantly by 5 days after trauma but was still present on day 14. Double labelling for TUNEL and GSA I-B_4, TUNEL and GFAP or GSA I-B_4 and GFAP at 24, 36 and 48 h after TBI showed that most of apoptotic cells were GSA I-B_4(-) and GFAP(-) and there was no collocalization for GSA I-B_4 and GFAP (Fig. 2). Distribution patterns of TUNEL(+) apoptotic cells, GSA I-B4(+) microglia and GFAP(+) astrocytes showed similar topography but a delayed time course with 12 hrs for microglia and 24 hrs for astrocytes compared to maximal expression of TUNEL(+) apoptotic cells (Fig. 3). Immunopositive cells for IL-1ß and IL-18 were first detected at 12 h after TBI at trauma site and in distant cortical areas: thalamus, striatum and white matter tracts with a peak at 24-36 h for IL-1ß(+) cells reached and at 36-48 h for IL-18(+) cells. Thereafter, both immunopositivities declined to insignificant levels by day 5-14 following trauma. Levels of IL-1ß and IL-18 mRNA were significantly elevated in thalamus and cortex ipsilateral to trauma site at 6 h and remained high for up to 24 h for IL-1ß and 72 h for IL-18. Densitometric analysis of Western blots for IL-1ß and IL-18 proteins revealed a significant increase of both proteins at 6 hours. IL-1ß protein decreased by 72 h, whereas IL-18 protein levels remained high for 3 days after the insult (data not shown). A significant decrease of IL-18 protein back to normal levels was observed by 7 days after trauma.

DISCUSSION

Hier we present evidence that TBI-induced microglial/astroglial activation is accompanied by increased expression of pro-inflammatory cytokines IL-1ß and IL-18. The initial upregulation of their mRNA and protein levels is followed by presence of a large number of immunopositive cells at the impact

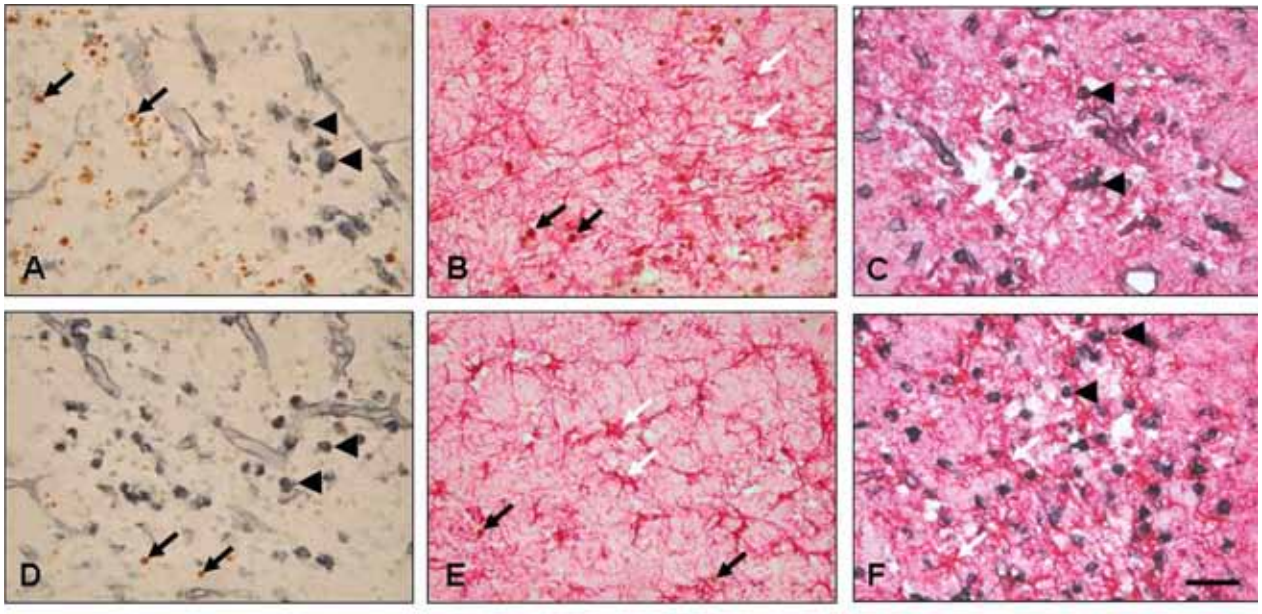

Fig. 2. Double immunostainings for TUNEL (black arrows) and GSA I-B4 (arrowheads (A, D), TUNEL and GFAP (white arrows) (B, E) or GSA I-B4 and GFAP (C, F) in thalamus (A, B, C) and capsula interna (D, E, F) 36 hrs after trauma to 7-day-old rat brain, TUNEL(+) cells - DAB substrate, GSA I-B4(+) microglia - SG substrate, GFAP(+) astrocytes - VNR substrate, bar in F valid for A-E=50 μm

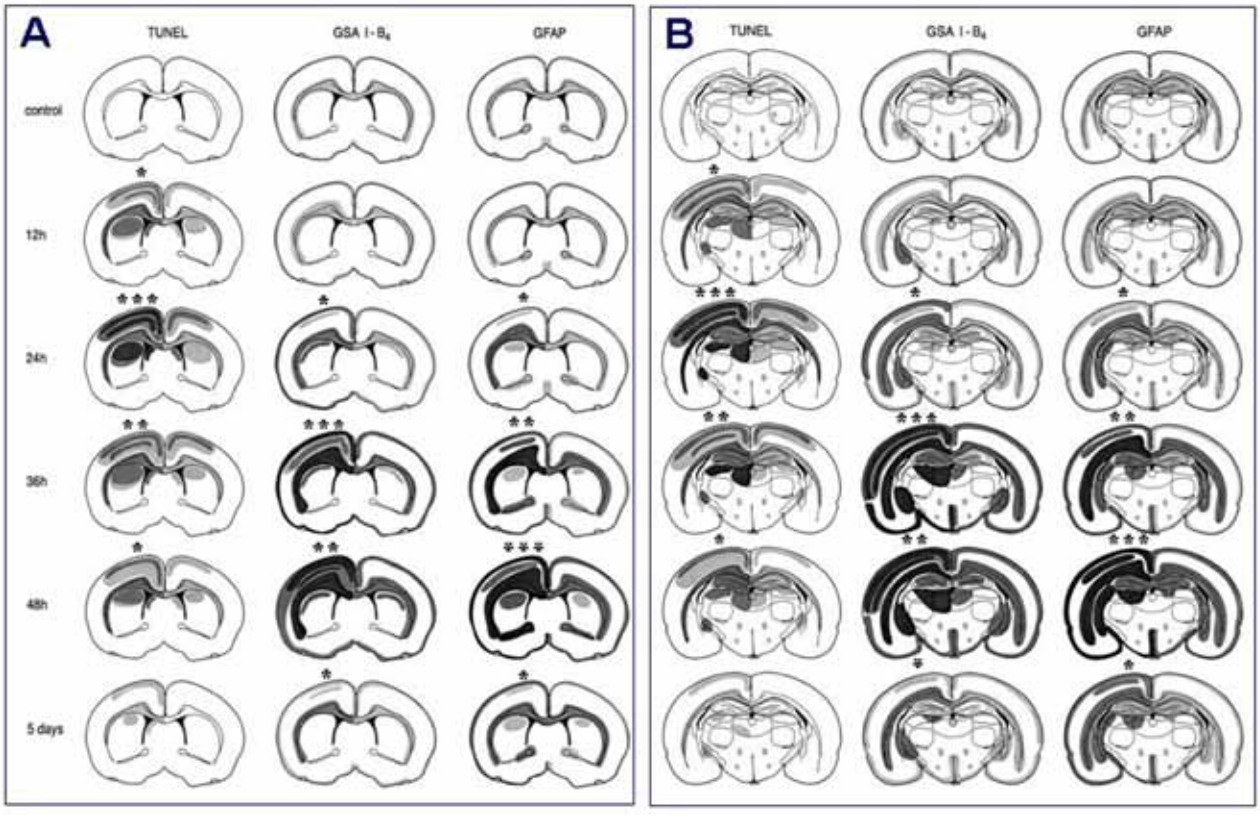

*Fig. 3. Distribution patterns of TUNEL(+) cells, GSA I-B4(+) microglia and GFAP(+) astrocytes in controls, 12, 24, 36, 48 hrs and 5 days after trauma in two coronal sectional levels at midstriatum (A) and midthalamus (B), * demonstrative expression, ** high expression, *** very high expression*

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site and in areas with pronounced apoptotic cell death. IL-1ß was more strongly expressed at trauma site, whereas IL-18 was mostly found in areas of apoptotic neurodegeneration over a longer time period as compared to Il-1ß. Some IL-1ß(+) and IL-18(+) cells were most probably blood-derived neutrophils and monocytes/macrophages at the injury site, as well as activated local microglia, reactive astrocytes and endothelial cells in the regions of secondary degeneration. Our data are more suggestive for a beneficial role for both interleukins as mediators of cellular interaction in the injured immature brain.

We registered an increased number of both main types of microglia normally present in small amount in the developing brain. The amoeboid microglia were localized mainly in the white matter, whereas resting or transitional phenotype microglia were found in the gray matter. Trauma induces a strong and rapid increase in the number of the amoeboid form, probably due to hyperplasia and transformation of resting microglia into reactive and phagocytic cells. Rapidly after trauma, we detected a large number of lectin(+) amoeboid and highly ramified transitional microglia together with ED1(+) blood-derived macrophages engulfing cellular debris in the peritraumatic region in close association with the area of cortical necrosis. We consider that reactive microglia are not responsible for initiation of apoptotic neurodegeneration but eventually they might contribute to its sustaining. Activated microglia may entertain the apoptotic process by secreting neurotoxins and other promoting factors, including cytokines (16). Accumulating data strongly suggest a neuroprotective role of microglia in acute CNS injuries as opposed to chronic CNS diseases with slowly progressive neurodegeneration (15). Activated microglia are known as secretors of astroglia-stimulating factors, whereas astroglia produce mitogenic factors and other substances modulating microglial inflammatory response (10). Recent evidence indicates that astroglial reactivity promotes CNS recovery and repair (9), as astrocytes produce a range of trophic factors. This cellular interaction is suggestive for a beneficial glial effect after TBI. In our study, we found a longer lasting astrocytic activation still present on day 14 after trauma as compared with shorter effects of microglia and interleukins. This is highly indicative for a possible involvement of reactive astrocytes in reparative processes at such longer time points after trauma than in destructive events.

CONCLUSIONS

In the developing brain, trauma provokes neuronal apoptosis leading to a strong activation of both microglia and astroglia. Microglial response is closely followed by post-traumatic reactive astrogliosis. Glial activation results in a local and systemic increase of IL-1ß and IL-18 pro-inflammatory cytokines. These processes might exacerbate post-traumatic apoptotic neuronal death during the early postnatal period but they might also promote tissue repair. Revealing the precise role of both glial cell types to progression of apoptotic neurodegeneration, inflammation and tissue repair in the immature brain may provide useful information for treatment of pediatric brain-injured patients.

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