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ORIGINAL ARTICLE

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Neuronal apoptosis following human brain injury

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Abstract Neuronal apoptosis has been investigated in paraffin-embedded brain tissue from 103 individuals who had sustained blunt head injury by use of the in situ nick translation (ISNT) technique. In order to provide reliable data for a forensic wound age estimation, a quantitative morphometric analysis was performed. Apoptotic neuronal cells could be detected in a cortical contusion with a wound age of 45 min at the earliest and in the majority of the cases with postinfliction intervals up to 2 weeks, numerous ISNT-positive cells were found adjacent to the traumatically injured area. The presented data indicate that neuronal apoptosis peaks at about 1 day and persists for at least 22 weeks after blunt head injury. The time-dependent occurrence of apoptotic cells can contribute to a forensic timing of cortical contusions and complements other immunohistochemical parameters, especially in the early postinfliction interval.

Keywords Human brain injury · Apoptosis · Wound age

Introduction

Neuronal cell death after traumatic brain injury has traditionally been ascribed to cellular necrosis which occurs not only at the impact site but also as a result of secondary brain insults such as intracranial hypertension, hypoxia or disturbances of microcirculation. In recent years it could be demonstrated that neuronal loss may also be due to programmed cell death [23], referred to as apoptosis. Apoptosis is an active, genetically programmed phenomenon of cell death that can be initiated or inhibited by a variety of stimuli [21]. It occurs in the developing brain [3, 10, 19, 30] and also plays an important role in neurodegenerative [27] and ischemic disorders [4] as well as in infectious diseases of the central nervous system [9, 22, 25]. Furthermore, apoptosis has been demonstrated in various animal models of traumatic brain injury [2, 7, 17, 18, 32] and there is also evidence of traumatically induced apoptosis in human brain [23]. Suárez-Peñaranda et al. demonstrated apoptosis in human skin injuries as an indicator of vital reactions in forensic autopsy cases [31]. The triggering of programmed cell death leads specifically to the formation of apoptotic bodies and intranucleosomal DNA cleavage, characterised by the generation of 3'-hydroxyl groups, which are often used as a marker of apoptotic cells. They can be detected by in situ labelling techniques [8, 20, 26] such as TUNEL (TdT-mediated dUTP nick end labelling) or ISNT (in situ nick translation).

Based on the findings of experimental studies demonstrating a temporal profile of apoptosis following traumatic brain injury in rats, the question arose whether time-dependent changes in the numbers of apoptotic cells can be detected in human brain tissue, possibly contributing to a forensic wound age estimation.

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Materials and methods

Specimens

Brain tissue with macroscopically visible cortical contusions was obtained at autopsy from 103 individuals who had sustained traumatic closed head injury. The brain injury was caused by car accidents (n=66), accidental falls (n=35) or homicidal blunt trauma (n=2). The individual age ranged between 6 and 81 years (average age 44 years). All individuals with a survival period up to 3 weeks died from cerebral dysregulation caused by neuronal damage, while in the remaining cases (n=6) secondary complications such as pneumonia (n=4) or lung embolisms (n=1) were found. One patient died from acute coronary insufficiency. Neither secondary haemorrhages, disturbances of blood coagulation which might influence the wound healing process nor previous CNS pathologies were evident according to the clinical data. Furthermore there were no clinical or morphological indications of intravital interruption of intracerebral circulation for a longer period of time. The survival period (wound age) ranged between a few minutes and 30 weeks and the post-mortem interval did not exceed 3 days.

At autopsy tissue samples about 3 cm^3 in size, were collected from the injured cortex (impact site) as well as from an area of the

contralateral cortex which did not show any focal lesions. Cortical tissue from 40 individuals without head injury who died of acute cardiac arrest (n=20), traumatic asphyxia (n=3), pneumonia (n=9) or lung embolisms (n=8) served as controls. The individual age of the control group ranged between 8 and 78 years and the mean age was 47 years.

After fixation in 4% PBS-formaldehyde solution for a maximum of 24 h the tissue samples were embedded in paraffin and sections (3–5 µ) were stained with haematoxylin and eosin (HE). Specimens with signs of autoloytic changes such as post-mortem cell shrinkage or diminished nuclear stainability in HE stained preparations were excluded.

ISNT technique

Staining of DNA fragmentation and apoptotic bodies was performed on paraffin sections using the in situ nick translation technique (ISNT). Briefly, deparaffinised and rehydrated paraffin sections were incubated with proteinase K (20 µg/ml) for 15 min at room temperature. After being rinsed with distilled water, endogenous peroxidase was quenched in 3% hydrogen peroxide for 10 min. The slides were rinsed in distilled water and then equilibrated in nick buffer (Tris $MgCl_2$, ß-mercaptoethanol, BSA 20 mg/ml, distilled water) for 10 min at room temperature. The ISNT was employed by incubating the slides with dNTPs and biotinylated 7-dATP diluted in nick buffer for 65 min at 37°C. The slides were then rinsed in terminating buffer (0.3 mol/l NaCl and 0.03 mol/l Na citrate) for 15 min at room temperature. After being washed in PBS, slides were incubated with extravidin-peroxidase (1:50) for 30 min at room temperature. The development was performed using AEC as substrate. Slides were subsequently counterstained with haemalaun, washed and mounted. Specificity of ISNT reactivity was confirmed by human epidermis and lymph node sections. Negative controls were performed with uninjured human brain tissue and by incubating in nick buffer without dNTPs and biotinylated 7-dATP.

Histological evaluation

Neuronal cells, identified by their cytological features and lacking positive reactions for GFAP, vimentin, tenascin or α1-antichymotrypsin in immunostained serial sections, were evaluated. Only cells showing a distinct nuclear staining by ISNT were referred to as "apoptotic cells" [28].

The numbers of apoptotic cells were counted in microscopic preparations from the lesion site, as well as from the contralateral cortex, assisted by an automatic image processing and analysis system (LEICA QWin) and using a field of view measuring frame of 0.125 mm^2 in size (objective 16/0.40, ocular ×10). The average number of positive stained cells defined as the "score" was determined within four measuring frames in the area surrounding the cortical lesion as well as in four randomly selected cortical areas of the uninjured contralateral cortex.

Results

Uninjured brain tissue

Apoptotic cells could not be detected in uninjured brain tissue from individuals without head injury (control group).

Traumatically injured brain tissue

Various apoptotic cell types were obvious under light microscopy:

1. Round-shaped apoptotic cells were regularly localised adjacent to the damaged area in cortical contusions older than 3 days (Fig. 1). The majority of these cells showed MIB-1 expression, as described for cerebral macrophages following human brain injury [14]. Some of the round-shaped cells could be labelled with the antibody against UCHL-1, which specifically recognises a subpopulation of T-cells with both CD4 and CD8 subsets [15], whereas an expression of glial markers (GFAP, tenascin, α1-antichymotrypsin) could not be observed.
2. Cells which could be identified as neurons by their morphological features (cell shape), lacking evidence of positive reactions for glial markers (GFAP, tenascin, α1-antichymotrypsin) in immunostained serial sections (Fig. 2). In order to obtain further information on the course of neuronal apoptosis, only this cell type was considered for the histological evaluation.

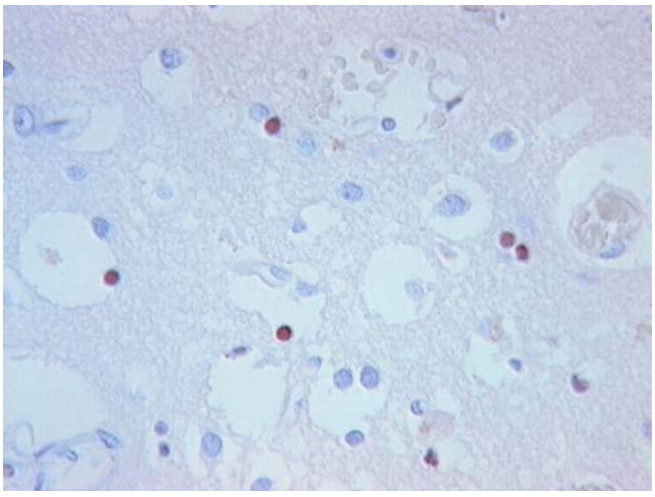

Fig. 1 Round-shaped cells adjacent to traumatically injured human cortex stained by use of the ISNT technique (post-infliction interval 12 days, ×280)

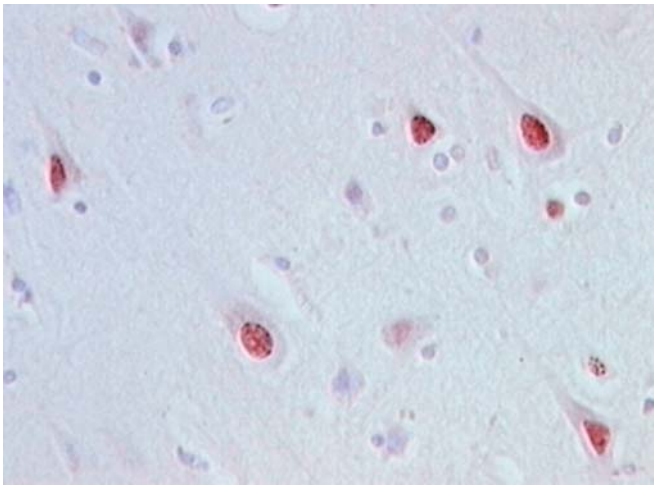

Fig. 2 Neuronal cells in the area surround a cortical contusion with a wound age of 2 days showing a distinct nuclear staining by ISNT (×280)

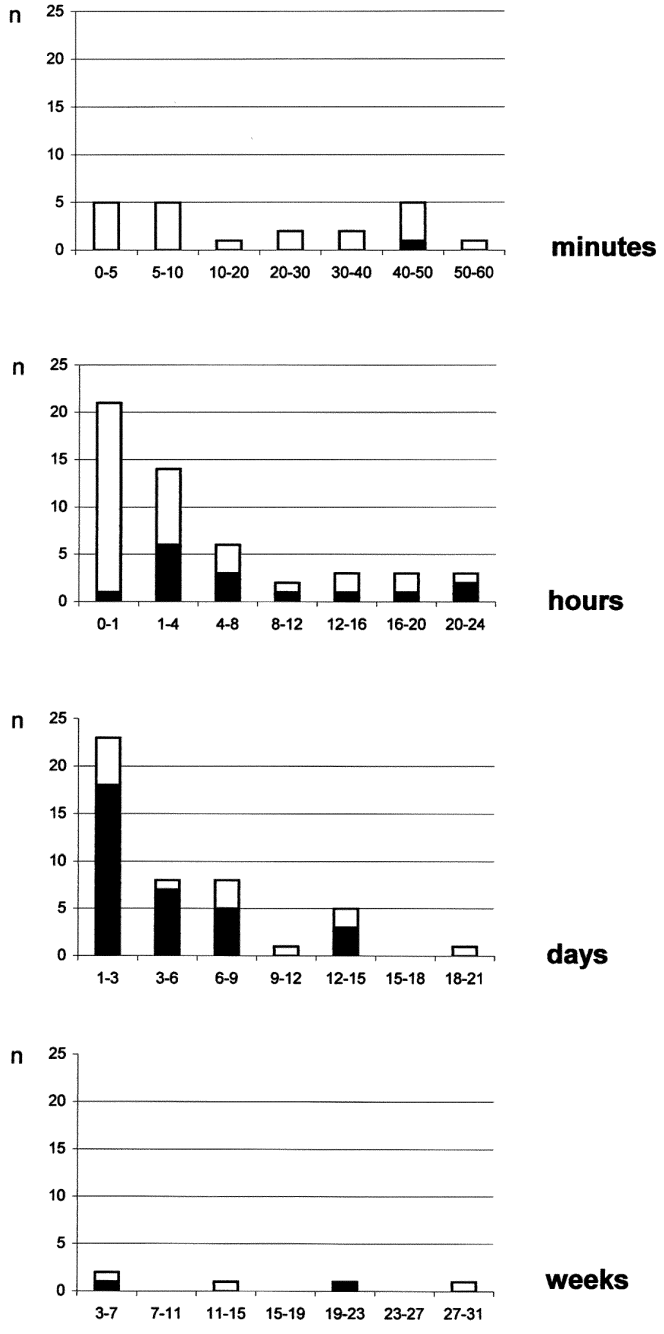

Fig. 3 Numbers of cases with apoptotic neurons adjacent to the cortical lesions, related to the wound age (*shaded area*)

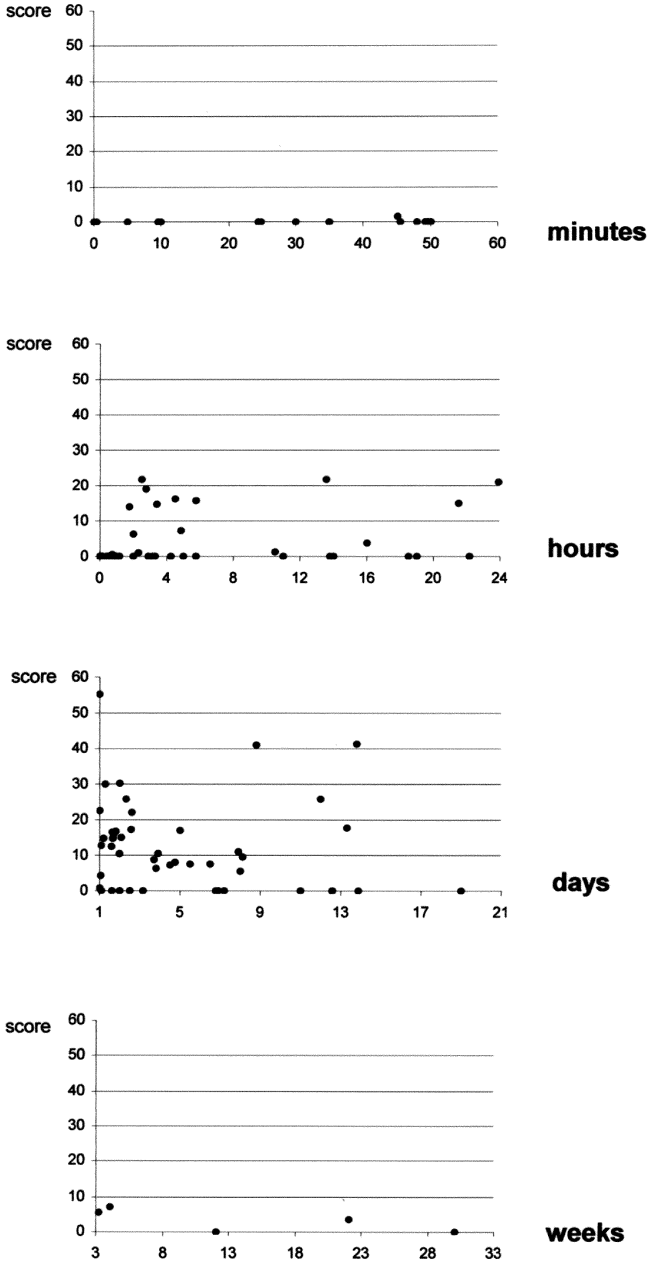

Fig. 4 Average numbers (score) of ISNT positive neuronal cells in four microscopic fields sized 0.125 mm^2 adjacent to cortical contusions

The earliest positive ISNT reaction was observed in a cortical contusion with a postinfliction interval of 45 min. In this case a few neuronal cells adjacent to the cortical lesion showed a distinct nuclear reaction (score 0.5), whereas apoptotic cells could not be detected in the tissue section from the uninjured contralateral cortex.

During the first day after the trauma, increasing cell numbers were found at the lesion site. In 14 out of 31 cases (45%) with a wound age of 1–24 h, apoptotic cells could be detected at the lesion site with average cell numbers up to 21.75 (wound age 2.5 h and 13.5 h). The maximum average number of apoptotic cells was found in a cortical contusion with a wound age of 1 day (score 55.25). In the postinfliction interval from 1–14 days, 33 out of 45 cases (73%) showed a positive ISNT reaction at the lesion site and in about 27% of these cases the apoptotic cell numbers score had mean values in excess of 20 cells/microscopic field. In cortical contusions older than 2 weeks and up to 22 weeks after the trauma, a positive ISNT reaction could be observed in 3 out of 6 cases and the maximum score was 7.25.

In the postinfliction interval between a few minutes and 30 weeks, the majority of cases (72%) with a positive ISNT reaction at the lesion site showed apoptotic neurons

Table 1 Frequency of cases showing a positive ISNT reaction adjacent to the cortical contusions as well as in the contralateral cortex related to the different states of wound healing

Wound age	*n*	Cortical contusion		Contralateral cortex	
		Positive	max. score	Positive	max. score
0–1 h	21	1 (5%)	0.5	0	–
1–24 h	31	14 (45%)	21.75	11 (35%)	13.5
1–14 days	45	33 (73%)	55.25	24 (53%)	41.75
2–30 weeks	6	3 (50%)	7.25	2 (33%)	3.25

also in tissue sections from the contralateral cortex. However, in most of these cases the average cell numbers were lower in uninjured brain tissue than in the areas adjacent to the focal lesion.

The results are demonstrated in Figs. 3 and 4 and summarised in Table 1.

Discussion

Apoptotic cells can be identified in microscopic preparations by different histochemical methods based on in situ labelling of free 3'-hydroxyl groups generated by intranucleosomal DNA cleavage. In this study the ISNT technique was used to identify apoptotic cells in traumatically injured human brain tissue. In order to obtain information on the course of neuronal apoptosis only neuronal cells showing a distinct nuclear ISNT reaction were considered for the quantitative histological evaluation.

Apoptotic neuronal cells could be detected in a cortical contusion with a wound age of 45 min at the earliest and in the majority of cases numerous ISNT-positive cells were found adjacent to the traumatically injured area up to 2 weeks after the injury. The presented data indicate that neuronal apoptosis peaks at about 1 day and persists for at least 22 weeks after the blunt head trauma. Apoptosis induced by trauma has also been found in experimental studies. However, there are considerable differences concerning the temporal profile which is thought to be influenced by the method producing the brain injury in animals [17]. The demonstration of apoptosis following cortical impact injury in rats provided data for the presence of apoptotic cells at 6 h, 24 h and 2 weeks after the trauma [5, 6]. In a weight dropping model of head trauma, apoptosis could be detected during the postinfliction interval between 3 h and 10 days [26]. The results of fluid percussion injury in rats [29] demonstrated that the number of apoptotic cells in the ipsilateral cortex reached a maximum at 24–48 h after the trauma. Kaya et al. [17] reported a temporal profile of apoptosis after cortical impact in rat brain with a progressive increase in the number of apoptotic cells from 1 h to 2 days, and a following decrease from 2 to 14 days post-injury.

Human brain tissue was investigated by Ng et al. for the first time [23]. In contrast to the findings of the present study, these authors reported the occurrence of TUNEL-positive cells in clinically collected material from head trauma patients as early as 2 h after the trauma and up to 192 h post-injury. The different temporal profile of apoptosis in cortical contusions may be due to the comparatively small number of cases in that study (n=11), whereas the present study included 103 cases with blunt head injury. In accordance with Ng et al., this study revealed evidence of apoptosis both in the area adjacent to the cortical contusions and in tissue samples from the contralateral cortex showing no traumatically induced focal lesions. These findings may possibly result either from diffuse axonal injury as a primary lesion or from secondary lesions after the head injury, such as posttraumatic ischemia due to brain swelling [23].

With regard to the time-dependent ISNT reactivity presented in this study, the detection of neuronal apoptosis is thought to be of value in the forensic wound age estimation and complements other immunohistochemical parameters which indicate neuronal damage [24] or inflammatory [15], glial [13, 14, 16] or vascular [11, 12] reactions. Although the results showed an interindividual variability, as principally expected in studies dealing with biological processes, the detection of neuronal cells seems most promising for the timing of cortical contusions, particularly in the early stage of wound healing. However, it must be noted that neuronal apoptosis is not specific for traumatic brain injury but can also be induced by a variety of other disorders in the central nervous system. Taking these aspects into consideration it can be concluded that the detection of apoptotic neuronal cells adjacent to cortical contusions by use of the ISNT method indicates a wound age of at least about 45 min, if other disorders of the central nervous system as a possible trigger of apoptosis are absent.

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