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TUNEL-positive staining of surface contusions after fatal head injury in man

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Abstract In frontal lobe contusions obtained post mortem from 18 patients who survived between 6 h and 10 days after head injury, DNA fragmentation associated with either apoptotic and/or necrotic cell death was identified by the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end labelling (TUNEL) histochemical technique. Additional histological techniques were also used to identify regional and temporal patterns of tissue damage. TUNEL-positive cells were present in both the grey and white matter of the contusion, where they peaked in number between 25 and 48 h, and were still identifiable at 10 days post injury. Fewer TUNEL-positive cells were observed in grey than in white matter; and most TUNEL-positive neurons in the grey matter demonstrated the morphological features of necrosis. However, the morphology of some TUNEL-stained neurons, and of TUNEL-stained oligodendroglia and macrophages in white matter was suggestive of apoptosis. Apoptosis was not seen in age- and sex-matched controls, none of whom had died from intracranial pathology or had pre-existing neurological disease. These findings suggest that multiple cell types in frontal lobe contusions exhibit DNA fragmentation and that both necrosis and apoptosis are likely to contribute to post-traumatic pathology. These findings provide further evidence that the observations made in animal models of traumatic brain injury have fidelity with clinical head injury.

Key words Human traumatic brain injury · TUNEL staining

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Introduction

The suggestion that after traumatic or ischaemic injury to the CNS neurons may die by necrotic and apoptotic pathways has stimulated extensive study into the mechanisms that underlie the different types of cell death, and the proportion in which they may occur. Necrosis is usually the consequence of severe damage and is characterised by swelling of the cells, a lytic disruption of all membranes and inflammation. In contrast, apoptosis requires energy and protein synthesis and is characterised morphologically by condensation and fragmentation of heterochromatin and the formation of apoptotic bodies [18, 29]. Since these original observations it has become apparent that apoptosis is associated with brain development [28], is found in tumours [20], and may also contribute to the ultimate outcome in such diverse conditions as stroke, epilepsy [11], HIV infection [33] and various neurodegenerative diseases, including motor neurone disease [53], Huntington's disease [49], multiple system atrophy [39] and Alzheimer's disease [42, 46, 50].

Much information about the incidence of DNA fragmentation and its time course has been provided by laboratory studies in models of acute cerebral ischaemia [11, 31], intracerebral haematoma [36] and after trauma to either the spinal cord [16, 19, 30, 52], the brain [13, 15, 41, 51] or the optic nerve [6, 7]. A recent study has demonstrated that surgical excised contusions from head-injured patients admitted to a neurosurgical ITU exhibit TUNEL-positive cells [13]. The present study was undertaken to more completely evaluate the time course of TUNEL staining cells in contusions and their relationships with the various pathologies of traumatic brain injury. A preliminary study has already been presented [45].

Table 1 Clinical and pathological details of head-injured cases [*PM* post-mortem, *R* right; *L* left, *B* bilateral, *(e)* evacuated, *Ass* assault, *RTA* road traffic accident, *NK* not known, *SDH* subdural haematoma, *EDH* extradural haematoma, *T* temporal, *F* frontal, *Mod* moderate, *ICP* intracranial pressure, + present, – absent]

Case	PM delay	Age (years)	Sex	Survival	Type of injury	Fracture of skull	Intracranial haematoma	Total contusion index	Diffuse axonal injury	Hypoxic brain damage	Swelling	Raised ICP	Others
1	118 h	58	M	6 h	Fall	+	SDH	32	–	–	–	–	Multiple injuries
2	5 h	39	M	12 h	Ass	–	B-SDH	8	–	Severe	R	+	Multiple injuries
3	66 h	62	M	12 h	Fall	+	L-SDH	30	1	Severe	L	+	Carcinoma of colon
4	75 h	61	F	20 h	RTA	+	R-EDH, B-SDH	34	–	Severe	R	+	–
5	22 h	66	M	21 h	RTA	+	L-SDH(e)	34	–	Mod	–	+	–
6	196 h	46	M	23 h	Fall	+	R-SDH(e)	6	–	Severe	B	+	–
7	68 h	75	F	24 h	Fall	+	L-SDH	27	–	–	–	+	–
8	12 h	60	F	26 h	Fall	+	R-RSDH(e)	16	–	Severe	R	+	Cirrhosis of liver
9	113 h	44	M	32 h	Fall	+	R-T(e)	13	1	Severe	B	+	–
10	47 h	20	M	36 h	Fall	+	–	16	1	Severe	B	+	Multiple injuries
11	117 h	41	M	41 h	Fall	+	R-SDH	24	–	Severe	R	+	Multiple injuries
12	153 h	46	M	48 h	Fall	+	–	6	1	Mild	–	–	Fracture of spine. Pulmon. embolism
13	191 h	55	M	4 days	NK	+	B-SDH	4	1	–	–	+	–
14	6 h	51	M	4 days	Ass	+	–	32	1	Severe	–	+	–
15	73 h	75	M	6 days	Fall	+	L-F, RT	16	1	–	–	–	Ruptured saccular aneurysm
16	19 h	60	M	9 days	Fall	+	R-F, LF	24	–	Mild	R	+	–
17	79 h	55	F	10 days	Fall	–	R-SDH	8	1	–	–	+	–
18	53 h	68	M	10 days	Fall	+	R-SDH, R-T	14	1	Mild	R	+	–

Patients and methods

Head-injured patients

Eighteen cases with subfrontal contusions (14 male and 4 female aged between 20 and 75 years; in 13 the cause of injury was a fall, in 2 a road traffic accident, in 2 an assault and in 1 it was not known) were examined. Details are provided in Table 1. The cases were divided into three groups based on the length of survival: group 1 (cases 1–6) with a survival between 5 and 23 h (average 16 h), group 2 (cases 7–12) with a survival of between 24 and 48 h (average 34.5 h) and group 3 (cases 13–18) with a survival of between 4 and 10 days (average 7 days). The interval from death to post mortem in the 18 cases ranged from 5 hours to 9 days (average 82.5 h).

Table 2 Controls

Case No	PM delay	Age (years)	Sex	Cause of death
1	9 h	64	M	Pneumonia: carcinoma of colon
2	12 h	52	M	Heart failure
3	23 h	20	M	Aspiration
4	28 h	57	F	Sudden unexplained death
5	34 h	62	F	Pneumonia
6	47 h	42	M	Drowning
7	73 h	75	F	Heart failure
8	94 h	38	M	Pneumonia: alcohol abuse
9	105 h	52	M	Pneumonia

Control cases

There were nine non-head injured patients from whom the subfrontal cortex was used as matched controls (six male and three females aged between 20–75 years) with post-mortem interval between 9 h and 5 days (average 49.5 h). Details are provided in Table 2. In none of the cases was the cause of death due to intracranial pathology. In one case there was a clinical history of pre-existing epilepsy and in some there may have been an element of agonal hypoxaemia.

Neuropathology

Brains were fixed in 10% formol saline for a minimum of 3 weeks prior to dissection, after which a full macroscopic and microscopic examination was undertaken in each case [2]. Contusions were assessed semi-quantitatively using the total contusion index (TCI). The TCI takes into account the depth and extent of surface contusions in various parts of the brain: zero means that there were no contusions, a TCI in the 20s is indicative of moderately severe contusions, while one of more than 37 is indicative of severe contusions [3]. Diffuse axonal injury (DAI) was graded as described previously [4]; only cases of grade 1 – least severe, were seen in this series in which there was widespread axonal damage in the corpus callosum as well as in the white matter of the cerebral hemispheres and in the brain stem. The severity of ischaemic brain damage – largely in the neocortex – was also graded. The damage was classified as mild if there were 5 or fewer small ischaemic lesions in the brain, moderate when the lesions were limited to arterial boundary zones, singly or in combination with subtotal infarction in the distribution of the cerebral arteries, or severe when the lesions were diffuse, multi-focal and large within the distribution of arterial territories [27]. Only haematomas thought to be suffi-

ciently large to act as significant intracranial expanding lesions (more than 35 ml) were recorded. Thus, a thin film of blood in the subdural space or small intracerebral haemorrhagic lesions were not recorded as haematomas. The criterion of pressure necrosis in one or both parahippocampal gyrus was used as evidence that the intracranial pressure after injury had been high as a result of a supratentorial expanding lesion [1].

From each of the head-injured cases a 1.0-cm-thick block of left-sided contused sub-frontal cortex was taken. Blocks from the same brain area were taken from the control cases. All the blocks were processed in a VIP tissue processor (Bayer Diagnostics) using a 60-h cycle.

TUNEL histochemistry

Sections (8 μm thick) were TUNEL stained as described previously [15, 21, 41]. Two deparaffinised wax-embedded sections from each block were treated with proteinase K (20 μg/ml in distilled water) for 20 min at 37 °C and subsequently washed four times for 1 min each in phosphate-buffered saline (PBS). Sections were then treated with a solution of 3% hydrogen peroxide in methanol for 5 min. After further washes in PBS, the sections were incubated in terminal deoxynucleotidyl transferase (TdT) buffer (30 mM TRIS pH 7.2, 140 mM sodium cacodylate, 1 mM $CoCl_2$) for 2 min. The slides were then incubated at 37 °C in TdT (25 U/ml) and UTP (50 nmol) in TdT buffer for 1 h. The sections were rinsed twice for 5 min each in 2 × SSC buffer (300 mM NaCl, 30 mM sodium citrate, pH 8.10) followed by three 1 min washes in PBS. Horseradish-peroxidase-streptavidin (1:200 Dako Ltd., High Wycombe, UK) diluted in 0.1 M TRIS pH 8.5, 50 mM NaCl, 4 mM $MgC1_2$ with 0.5% Tween-20 was then applied for 60 min at room temperature. The sections were then rinsed three times for 1 min each with PBS after which the chromogen, amino-ethyl-carbazole (Vector Laboratories, Peterborough, UK), was applied for 10 min prior to further rinses. The sections were then cover slipped with a permanent aqueous mounting medium (Supermount, A. Menarini Diagnostics Ltd, Wolverhampton, UK). Each batch stained contained both positive tissue from glioblastoma multiforme and negative control material in which TdT and UTP were excluded.

TUNEL positivity was determined by two observers (F.S. and D.I.G.) "blind" to the survival of the case material. It was not possible to be "blind" to material from the head-injured cases because of the ease by which the contusions could be identified. After an initial period of learning a high degree of intra- and inter-observer reliability was established – greater than 95%.

Neurohistology

Paraffin-embedded sections, adjacent to those in which TUNEL histochemistry was undertaken, were stained (1) with hematoxylin and eosin (H&E), (2) by Luxol fast blue-cresyl violet (LFB/CV), (3) for reticulin, (4) by van Gieson for collagen, and (5) by the Perl method for haemosiderin. In addition, overnight immunohistochemistry at 4 °C was undertaken for glial fibrillary acidic protein (GFAP; polyclonal antibody, Dako, 1:2,000) to detect astrocytes, for microglia and macrophages (CD68 monoclonal antibody, Dako, 1:200), and for amyloid precursor protein (APP; monoclonal antibody, Boehringer Mannheim, 1:3,000) to detect axonal injury. All three antibodies were detected using the ABC kit (Vectastain, Vector Laboratories, Peterborough, UK).

Display and quantitation of data

Scale diagrams of the subfrontal region of each head-injured and control case were drawn, upon which the contusions, associated cellular responses and the distribution and number of TUNEL-positive cells were superimposed using a Kontron electronic image analysis system (Vidas). In this way it was possible to display the number of TUNEL-positive cells in grey and white matter related to the area of contusion.

Using established criteria [40], TUNEL-positive cells were separated into two categories: type I which had the morphological appearance of non-apoptotic cells, and type II, which had the histological features of apoptosis. The absolute number of TUNEL-positive cells per unit area of cortex and white matter in the paraffin sections cut from the contused frontal lobes of each case was counted using a calibrated eye piece graticule measuring 0.066 mm^2. The TUNEL-stained sections were interpreted and quantified as quickly as possible because of their tendency to fade.

Analysis

A two sample T test was used to assess any differences between the head-injured patients and the controls with respect to age, and the Mann Whitney test for survival and post-mortem interval. There was no statistical differences in these parameters between the test group and controls. Given the heterogeneity of the findings it was decided that the data should be displayed graphically and without formal analysis.

Results

Conventional neurohistology

Histologically, the contused areas appeared as areas of haemorrhage predominantly in the superficial layers of the grey matter: in some instances the contusion also affected the related white matter. In contrast, white matter distant from the contusion appeared normal (Fig. 1 A, B).

By 12 h post injury, it was possible to identify neuronal necrosis not only in grey matter immediately related to the contusions, but also more remotely in the cortex. In the H&E- and LFB/CV-stained sections the appearances were those of ischaemic necrosis in which the affected neurones, embedded in a finely vacuolated neuropil, were contracted with condensed triangular nuclei, eosinophilic cytoplasm (Fig. 1 A) and incrustations. By 48 h there were areas of cortical infarction, and by 8 days there was a clear line of separation between organising contused brain and adjacent normal tissue.

In all of the head-injured cases contusional damage was also seen in white matter adjacent to the contusions. Between 12 and 24 h there was some pallor of myelin staining in the LFB/CV preparations, indicative of oedema. Reactive astrocytes and microglia were first seen in the H&E-stained sections at 48 h and, with increasing survival times, there were microglia and macrophages in relation to the contused area and in related white matter.

Immunocytochemistry for GFAP showed limited changes by 12 h post injury, consisting of fibre-forming astrocytes around blood vessels, beneath the pia and scattered throughout white matter. By 48 h post injury, there was some accentuation of these appearances, and a particularly prominent astrocytic response was observed after 7 days post injury in areas immediately related to the contusion, but also more diffusely throughout white matter. In the short-surviving cases, CD68-positive staining was limited to a few microglia with some enhancement by 48 h. Between 4 and 10 days a prominent macrophage infiltrate

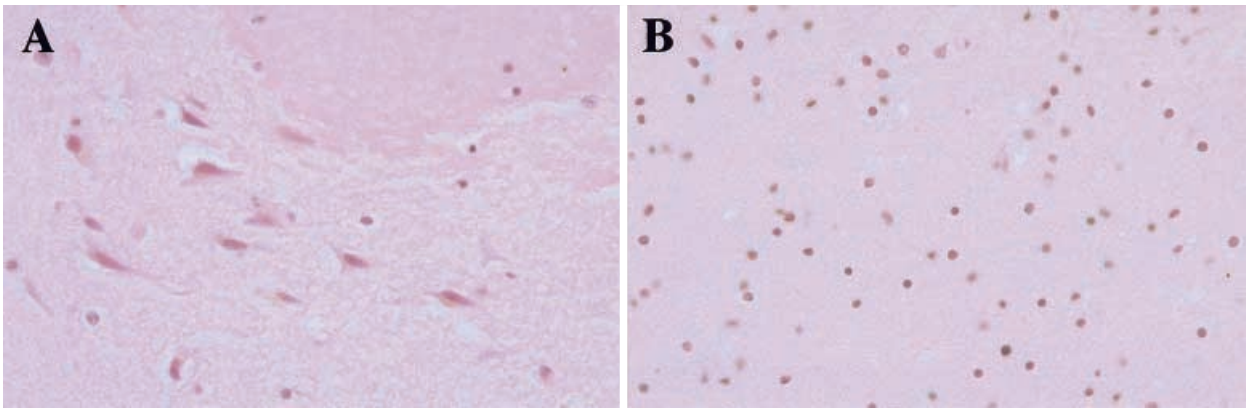


Fig. 1 A, B Surface contusion (survival 41 h). **A** There is haemorrhagic necrosi of the cortex. **B** The white matter adjacent to the contusion appears normal. **A**,**B** H & E × 2 μm

was seen at the margins of the contusions: some of them contained haemosiderin. A few macrophages were also present in the meninges and elsewhere in the tissue sections. Throughout the period of study the oligodendroglial nuclei were unreactive and apparently normally distributed. By 7 days there was some collagenous thickening of the meninges in both the reticulin and van Gieson preparations. APP-positive axons were seen at the margins of the contusions in the head-injured cases: the amount of immunoreactivity increased up to 24–48 h and subsequently plateaued.

TUNEL staining

In general two types of TUNEL-positive cells were observed [15, 41]. Type I had the morphological appearance of non-apoptotic cells that stained darkly but lacked nuclear condensation and fragmentation (Fig. 2 A, B). Type II or apoptotic cells were characterised by dark staining and occasionally fragmented nuclei with condensed chromatin (Fig. 2 C).

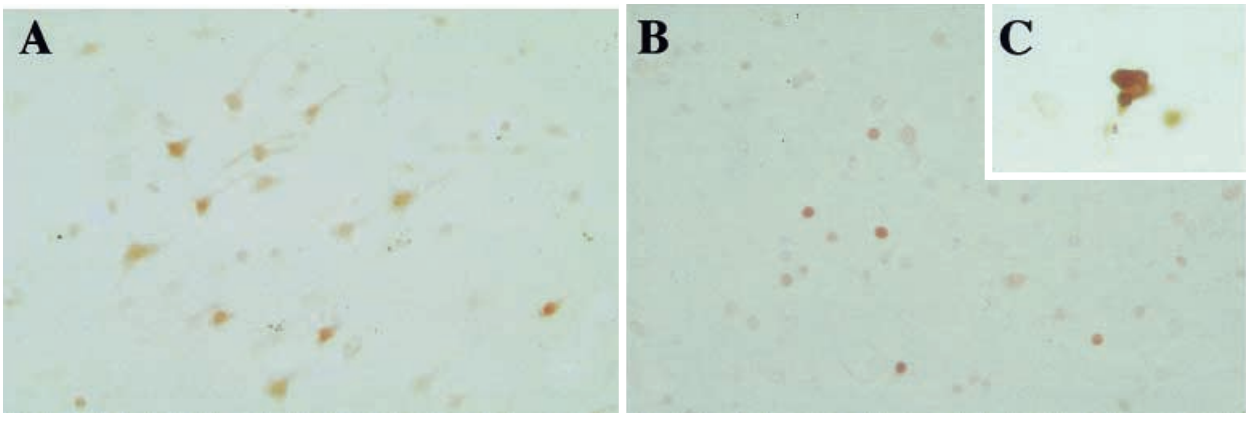
Fig. 2 A–C TUNEL staining (survival 41 h). **A** Type 1: the shape and size of the nuclear profiles clearly indicate their neuronal nature. **B** Type I: the nuclei in the white matter are smaller and have the appearances of glial cells. C Type II: the nucleus of this cell in the white matter has the morphological features of apoptosis. TUNEL staining, **A**,**B** × 200, **C** × 400

Distribution and quantitation of cellular changes

In all the head-injured cases TUNEL-positive cells were more numerous in white than grey matter. An example from each survival group is illustrated (Figs. 3, 4, 5).

Group 1 (5–23 h)

Serial sections from a representative head-injured patient from group 1 are shown in Fig. 3 to illustrate the relative distributions of various histopathological changes. The contusion from this head-injured patient (5 h survival), comprised a series of haemorrhages in both grey and white matter (Fig. 3 A). Foci of early neuronal necrosis were seen in the cortical grey matter (Fig. 3 B) immediately related to the haemorrhages and at this stage there was minimal reactivity of astrocytes (GFAP, Fig. 3 C) and no microglia (CD68, Fig. 3 D). In contrast, considerable numbers of TUNEL-positive cells were found in the white matter away from the immediate area of contusion (Fig. 3 E), although in the cortex there was an apparent relationship between the distribution of TUNEL-positive staining and the ischaemic necrosis of the contusion.



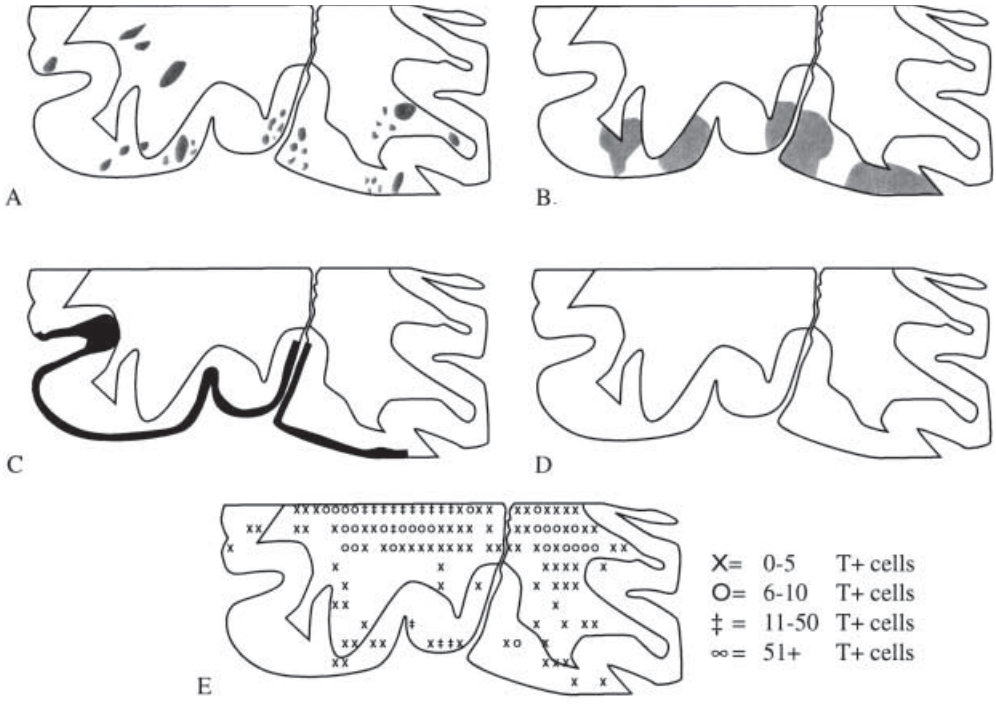


Fig. 3 Relationships between various cellular components in group 1 case (survival 5 h)

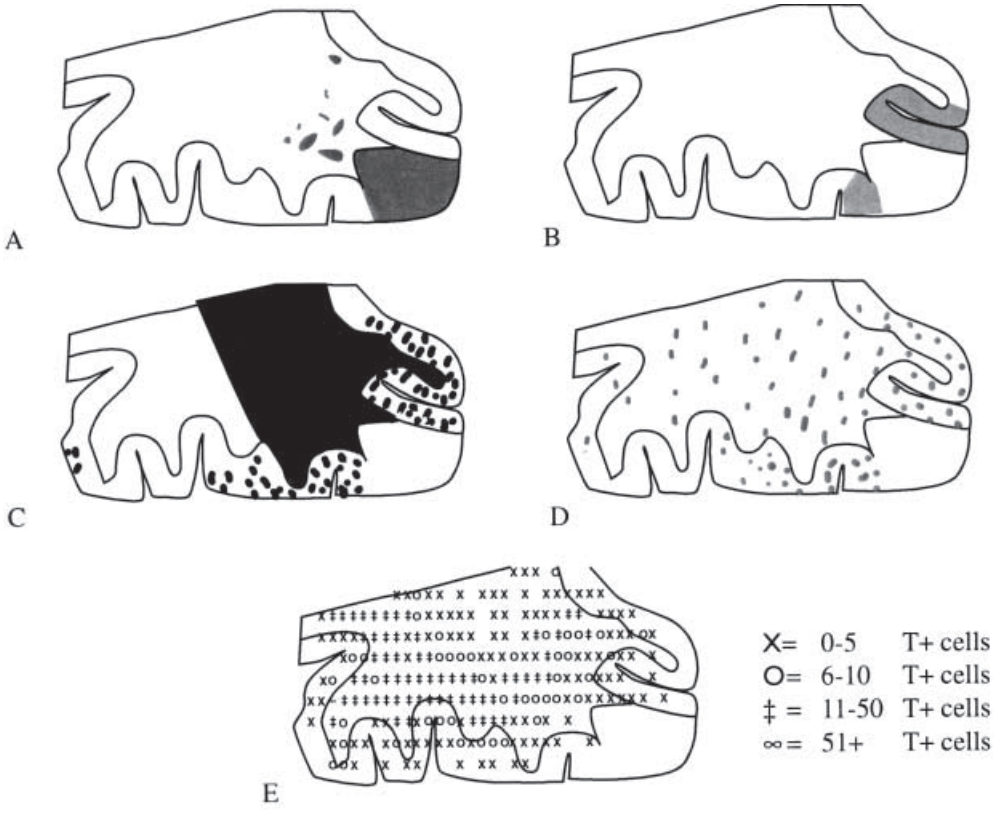


Fig. 4 Relationships between various cellular components in group 2 case (survival 48 h)

The number of TUNEL-positive cells in both grey and white matter increased from 5 to 23 h: Type I cells were present in cortex and white matter. Type II cells were uncommon in grey matter and were seen most frequently in white matter.

Group 2 (24–48 h)

Serial sections from a representative case in this group and the distribution of findings are shown in Fig. 4. The contusion (Fig. 4 A) and its associated necrosis (Fig. 4 B) are shown. At 48 h there were reactive changes in both as-

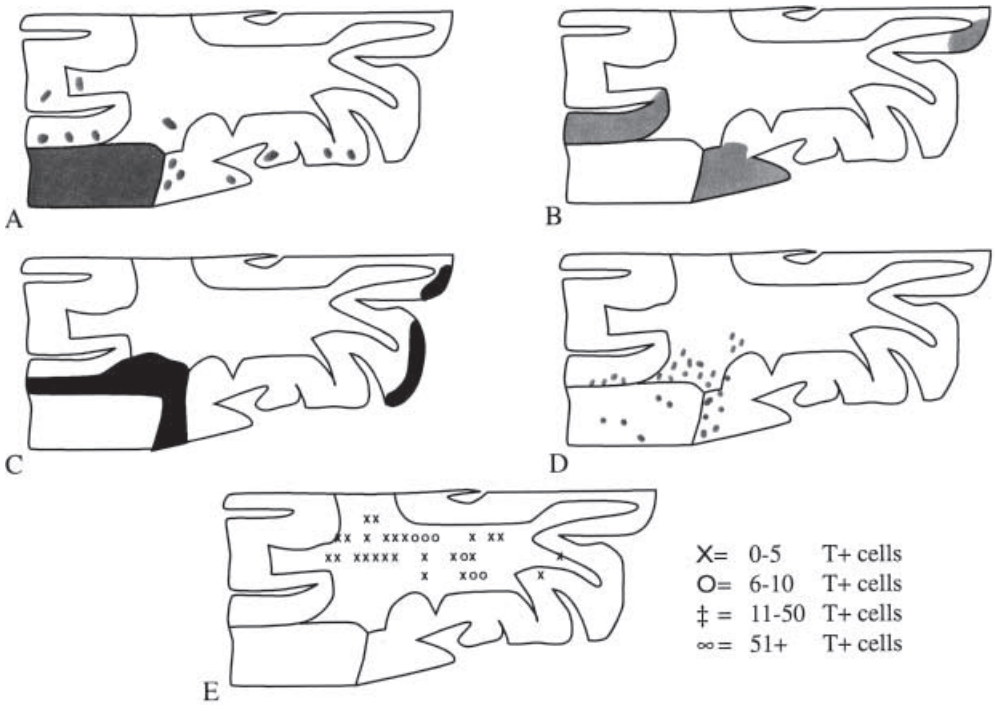


Fig. 5 Relationships between various cellular components in group 3 case (survival 8 days)

trocytes (GFAP, Fig. 4C) and microglia (CD68, Fig. 4D) especially in relation to the area of contusion (Fig. 4A, B). Large numbers of TUNEL-positive cells were also seen (Fig. 4E), more of which were located in white than in grey matter. Type I TUNEL-positive cells were present in both grey and white matter and in these cases type II cells were only seen in white matter.

Group 3 (4–10 days)

Serial sections were cut from a representative head-injured patient in this group and the findings are illustrated in Fig. 5. By this time the contusion was undergoing organisation (Fig. 5A, B) with margins comprising reactive astrocytes (Fig. 5C) and macrophages (Fig. 5D). Haemosiderin was present in macrophages. TUNEL-positive staining was no longer seen at the margins of the contusion in the cortex but was limited to adjacent white matter (Fig. 5E). Although fewer in number, TUNEL-positive cells were again predominant in white matter.

Quantitation of TUNEL-positive cells

Only the occasional TUNEL-positive cell was seen in the control material. TUNEL-positive cells were seen as early as 5 h post injury (Fig. 3E). They were maximum in number between 24–48 h (Fig. 4E) and could still be seen 8 days post injury (Fig. 5E). In all cases more TUNEL-positive cells were seen in white matter than grey. Type I TUNEL-positive cells were found in both grey and white matter and accounted for the great majority of the stained cells in the grey matter. Type II TUNEL-positive cells were also found in both grey and white matter, although they predominated in the latter. Of the TUNEL-positive cells in either grey or white matter only 5% had the morphological features of apoptosis.

Discussion

The nature and distribution of the various classical pathologies in traumatic brain injury are well known [26]. Contusions on the surface of the brain are considered to be one of the hallmarks of a head injury. They are a focal form of traumatic brain injury and occur at the instance of injury and, therefore, can be characterised with certainty from injury until death [3]. Histologically, contusions typically form through post-traumatic bleeding into affected tissues and are invariably associated with ischaemic necrosis, principally in grey but also in related white matter. With increasing survival the damaged tissue is resorbed and replaced by a shrunken scar that is usually pigmented by haemosiderin.

Contusional injury has been replicated in the laboratory in a variety of animals [24]. In the non-human primate it appeared that the extent of contusions was primarily determined by the immediate traumatic event, and that the depth of a contusion could increase over time [25]. More recently, it was reported that after fluid percussion-induced brain injury in the rat, the volume of cortical contusion increased with prolonged survival [8, 35, 38, 44]. Long-term changes have also been described after controlled cortical impact [17]. Such evidence, combined with anecdotal clinical observations, suggest that, in addition to the necrosis induced at time of acute brain injury, there may also be a series of cellular events with a more

protracted time course. One such process may be that of apoptosis [28]. Apoptosis has been described in the developing nervous system [9], after 'stroke' [11, 32, 34] and in various neurodegenerative diseases [28]. The first evidence of apoptotic cell death after experimental traumatic brain injury was provided by Rink et al. [41] using lateral fluid percussion in the rat. Using a combination of TUNEL staining and electron microscopy these authors were able to identify two types of TUNEL-positive cells. Type I had the features of necrotic cell death, while type II had the features of apoptosis (identical to the changes found in this study). In this rodent model TUNEL-positive cells were detected for up to 72 h after the initial injury, the longest period for which the animals were allowed to survive. Additional evidence for the involvement of apoptotic pathways was provided by gel electrophoresis of DNA extracted from injured brain and by electron microscopy. Subsequent studies have validated these observations [12, 14, 15, 51] and shown that apoptosis occurs as late as 2 months after experimental brain injury.

In a recent study of surgically removed frontal and temporal lobe contusions in a series of head-injured patients between 1 and 9 days post injury [13], evidence of DNA fragmentation with both apoptopic and necrotic morphologies was found. Using a technique similar to that described by Gavrieli et al. [21], we have also been able to achieve TUNEL-positive staining in relation to frontal contusions in patients who have survived for up to 8 days after injury. As reported by Rink et al. [41] and Conti et al. [15], it has been possible to identify two types of TUNEL-positive cells: type I that occur in grey and white matter and in the former occur mainly in neurons undergoing ischaemic necrosis, and type II that have the morphological features of apoptosis and occur principally in white matter. Such an interpretation is supported by sections stained with either H&E or combined LFB/CV in which it is easy to recognise the presence of ischaemic neuronal necrosis. The similarities between the observations made after lateral fluid percussion brain injury in the rat [15, 41] and those found in the current clinical study suggest that this experimental model does have fidelity with clinical head injury.

In the studies by Rink et al. [41] and Conti et al. [15] quantitative studies were undertaken in both grey and white matter from a number of brain areas. The greatest number of TUNEL-positive cells were observed in the contused cortex and sub-cortical white matter, ipsilateral to the site of impact; many fewer were seen in the equivalent areas of the contralateral hemisphere. Sampling from many brain areas was not undertaken in the present study and so a direct comparison with the rat model cannot be made. In contrast, the number of TUNEL-positive cells associated with frontal lobe contusions in man was much greater in the white than in the grey matter. Furthermore, the number of TUNEL-positive cells increased with survival and peaked between 25 and 48 h. Although the number of TUNEL-positive cells in grey matter decreased over the subsequent 10 days, there were a rather surprising large number of persisting TUNEL-positive cells in the longest surviving patient compared to the control case material.

Although double-labelling studies were not undertaken in the current study, the histological features of the type I TUNEL-positive cells suggested that the changes were taking place within neurons. In contrast, the type II cells appeared as either oligodendroglia or macrophages rather than astrocytes or endothelial cells. Such interpretation is in keeping with the recent observations [16, 30, 43, 52] that have described apoptosis in experimental models of spinal cord injury. In the spinal cord-injured rat, apoptotic oligodendroglia were found in the white matter from 6 hours to 3 weeks after injury. Similar changes were found within various degenerating fibre tracks in a group of spinal cord injured non-human primates [16]. Taken together these data suggest that apoptosis of glial cells, particularly in white matter, is a feature of traumatic CNS injury with a greater frequency and a more prolonged time course than neuronal apoptopic cell death in grey matter. Laboratory studies have shown that the post-mortem interval does not significantly influence the amount of DNA fragmentation [37].

Loss of oligodendroglia due to apoptotic mechanisms has been described in association with trauma-induced demyelination [16, 43]. However, even in multiple sclerosis [40] there is lack of conclusive evidence that oligodendroglia become depleted by apoptosis. This may in part have been due to difficulties in the labelling of adult oligodendroglia in formalin-fixed, paraffin-embedded material, although success had been achieved with pi-glutathione S-transferase immunohistochemistry [10, 47, 48].

Activated microglia and the formation of macrophages were features of the contused material and in most cases there appeared to be a relatively close association with TUNEL-positive cells. This microglial/macrophage response may reflect axonal or neuronal damage [22, 23], as characterised by regional increases in APP immunohistochemistry, or may be a response to the presence of the TUNEL-positive cells degenerating per se. Conversely, these activated microglia could contribute to the ongoing apoptosis of oligodendroglia in the white matter [5].

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