American Journal of Pathology, Vol. 147, No. 6, December 1995

Short Communication

Evidence of Apoptotic Cell Death after Experimental Traumatic Brain Injury in the Rat

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Apoptosis plays an important role in many developmental and pathological processes of the central nervous system. However, the role of apoptosis in traumatic brain injury has not been determined. Using the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end labeling (TUNEL) method, we detected many cells with extensive DNA fragmentation in different regions of the brains of rats subjected to experimental traumatic brain injury. Two types of TUNEL-positive cells were demonstrated by light and electron microscopy, including type I cells that displayed morphological features of necrotic cell death and type II cells that displayed morphological features of classic apoptotic cell death. TUNEL-positive cells were detectable for up to 72 hours after the initial injury. Gel electrophoresis of DNA extracted from affected areas of the injured brain containing both type I and II cells revealed only internucleosomal fragmentation at 185-bp intervals, a feature originally described in apoptotic cell death. These data suggest that apoptosis, in addition to necrotic cell death, occurs after traumatic brain injury, and that internucleosomal fragmentation of DNA may be associated with certain types of necrotic cell death. (Am J Pathol 1995, 147:1575–1583)

Widely distributed neuronal damage and neuronal loss have been well documented in the cerebral cortex, hippocampus, and thalamus of the rat central nervous system (CNS) during the first few hours after experimental traumatic brain injury (TBI). The acute phase of neuronal damage is followed by a delayed phase of neuronal loss in the affected areas within the first 4 weeks after TBI.[1–3] In the widely utilized lateral fluid percussion model of brain injury in the rat,[2,4] the pyramidal neurons of the hippocampal CA3 region and the dentate gyrus appear to be the most vulnerable areas. Indeed, protracted bilateral neuronal loss occurs in these two areas as a result of unilateral TBI that induces no detectable damage to the adjacent non-hippocampal and non-hilar neurons.[5] Significantly, the extent of neuronal damage in the hippocampus of this model of brain trauma closely correlates with the severity of the posttraumatic cognitive deficits, with persistent memory dysfunction,[5,6] and with the occurrence of seizures.[7] The mechanisms of neuronal death after TBI are not well understood, although necrosis, the end product of passive cellular damage, has been well documented in both clinical and experimental TBI.[1–3] It remains to be determined whether programmed cell death (PCD)[8] also occurs after TBI, and if it might contribute to the behavioral deficits that result from traumatic injury to the CNS.

The salient feature of PCD, in contrast to necrotic cell death, is its active nature, representing the initiation of a suicidal pathway, initiated intrinsically by

Supported in part by NIH grants CA-36245, AG-11542, NS-26818, NS-08803, and MH-43880.

Accepted for publication September 12, 1995.

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the cell. In many examples PCD is mediated by *de novo* gene expression as well as by translation- or transcription-dependent mechanisms.[9–12] In most systems where PCD has been studied, apoptosis represents the principal mechanism of cell death. For all practical purposes, apoptosis is regarded as the *sine qua non* of PCD.

Cells undergoing apoptosis display characteristic morphological and biochemical features including cell shrinkage, formation of apoptotic bodies, condensation of chromatin, nuclear fragmentation, and extensive internucleosomal DNA fragmentation. Recently, a novel technique, terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL),[13] has been used to detect *in situ* DNA fragmentation. Because TUNEL detects extensive DNA fragmentation, it is reasonable to characterize cells with extensive DNA fragmentation detected by TUNEL histochemistry as dying or dead cells, regardless of the mode of cell death. The implication of TUNEL positivity should be interpreted in conjunction with morphological data. In addition, internucleosomal fragmentation of genomic DNA at 185 to 200 bp intervals[8] represents a biochemical signature of apoptosis that presents characteristically as a DNA "ladder" in gel electrophoresis.

Apoptosis appears to play an important role during development of the CNS.[14,15] Recent evidence also identified apoptosis as an important component of degenerative processes such as retinitis pigmentosa,[16] experimental brain ischemia,[17] and brain tumor formation.[18–20] Overexpression of the endogenous protein Bcl-2 *in vivo* can prevent the naturally occurring apoptotic death of neurons,[21] whereas neurons induced to undergo apoptosis in culture (eg, by depriving them of nerve growth factor) can be rescued by resupplying nerve growth factor within about 12 hours after growth factor deprivation.[22] In contrast to apoptosis, necrosis is regarded as a form of cell death resulting from passive damage to the cells. Necrotic cell death is characterized at the morphological level by cell swelling, lysis, and disrupted cell membranes, as well as by randomized DNA fragmentation leading to a DNA smear in gel electrophoresis.[8] Necrosis is usually involved in pathological but not physiological processes.

To test the hypothesis that apoptosis might play a role in TBI, we sought to detect cells with the above-mentioned features of apoptosis in rats subjected to lateral (parasagittal) fluid percussion (FP) brain injury *in vivo* using a well characterized experimental paradigm that simulates human closed head injury.[2,4] In addition to morphological evidence of apoptosis, TUNEL histochemistry was used to identify DNA fragmentation *in situ* at both light and electron microscopic levels, whereas characteristic internucleosomal DNA fragmentation of apoptosis was demonstrated by DNA gel electrophoresis.[23]

Materials and Methods

Animals

Experimental FP brain injury was performed as described previously.[2,4] Briefly, adult male Sprague-Dawley rats (350 to 400 g) were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/kg) and placed in a stereotactic frame. A 5.0 mm craniotomy was made over the left parietal cortex and the animal was connected to the FP device. FP brain injury was generated by the rapid injection of a bolus of saline through the craniotomy, leading to a sudden increase of intracranial pressure. Rats were subjected to FP brain injury of mild (1.0 to 1.2 atm, n = 15) or moderate (2.0 to 2.4 atm, n = 15) severity and were sacrificed at 12, 24, 48, or 72 hours after brain injury (n = 3 for each time point). Sham controls (n = 3) were subjected to anesthesia and surgery without brain injury. The majority of brains were processed into paraffin blocks and sectioned for TUNEL staining (n = 27) after transcardiac perfusion of the animal with heparinized saline followed by 4% phosphate-buffered paraformaldehyde. Brains obtained at the 24 hour sacrifice time point after mild (n = 3) and moderate (n = 3) brain injury were snap-frozen without perfusion and stored at −70°C until DNA gel electrophoresis. These protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

TUNEL Histochemistry

TUNEL histochemistry was performed as described previously.[13] Briefly, 6 μm thick serial coronal sections were cut from the mid-hippocampus at anterior to posterior stereotaxic positions of −3.3 to −4.3,[24] affixed to slides by heating at 60°C for 15 minutes, deparaffinized, and rehydrated. After digestion with 0.02% trypsin in phosphate-buffered saline (PBS) at room temperature and extensive washes in PBS, the sections were incubated in buffer A (200 mmol/L potassium cacodylate, 0.025 mmol/L Tris, 0.25 mg/ml bovine serum albumin at pH 6.6) for 5 minutes. The sections were then incubated with the labeling solution containing TdT (0.3 U/μl, Boehringer Mannheim, Indianapolis, IN), biotinylated-16-dUTP (20 μmol/L, Boehringer Mannheim), 1.5 mmol/L co-

balt chloride in buffer A at 37°C for 60 minutes. The reactions were terminated by rinsing in buffer B (300 mmol/L sodium chloride and 30 mmol/L sodium citrate at pH 7). The sections were then washed with 0.01 mol/L Tris buffer at pH 7.5 and blocked with 10% goat serum in 0.01 mol/L Tris buffer for 15 minutes. For light microscopy, the labeled DNA fragments were visualized by incubating the sections with a 1:40 dilution of streptavidin-conjugated alkaline phosphatase (BioGenex, San Ramon, CA) followed by reaction with medium containing fast red as chromogen (Sigma Fast Kit, Sigma Chemical Co., St. Louis, MO). The slides were then washed, counterstained, and mounted in an aqueous medium.

Electron Microscopy

The TUNEL-labeling procedure for electron microscopy was identical to that used for light microscopy, except endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol before labeling and streptavidin-conjugated peroxidase was used in place of streptavidin-conjugated alkaline phosphatase. The sections were developed in substrate buffer containing diaminobenzidine and hydrogen peroxide as previously described,[25] and then treated with 0.1 mol/L cacodylate buffer, postfixed in 4% osmium tetraoxide, dehydrated, and embedded *in situ* in Polybed 812 Resin (Polyscience, Warrington, PA). Thin serial sections were cut and mounted on grids, stained with lead citrate, and examined with a Hitachi H-600 electron microscope.

Gel Electrophoresis

DNA end labeling and gel electrophoresis were performed as described previously.[23] Because this method adds only one radioactive nucleotide to each end of fragmented DNA, the effect of this procedure on the molecular weight of the labeled DNA is negligible. Briefly, frozen brain tissue was thawed, homogenized in buffer (0.1 mol/L NaCl, 0.01 mol/L EDTA, 0.3 mol/L Tris-HCl, 0.2 mol/L sucrose, 0.6% sodium dodecyl sulfate, pH 8.0), and incubated at 68°C for 60 minutes. Protein precipitation was facilitated by adding potassium acetate to a final concentration of 1.2 mol/L and incubation on ice for 60 minutes. The precipitates were spun down and the supernatants digested with DNAse-free RNAse (2 μg/ml) at 37°C for 60 minutes, extracted with phenol-chloroform, precipitated with isopropanol at −20°C overnight, dried, and redissolved in distilled water. For end labeling, 1 μg of purified DNA was incubated with 30 U TdT (GIBCO BRL, Gaithersburg, MD), 30 μCi of [α-^{32}P]ddATP (Amersham, Arlington Heights, IL), 10 μl of 5× concentrated buffer (Gibco BRL) and water in a final volume of 50 μl at 37°C for 60 minutes. The reaction was terminated by adding 2.5 μl of 0.5 mol/L EDTA, and the labeled DNA was precipitated twice by ethanol and 10 mol/L ammonium acetate with 50 μg of yeast tRNA as carrier. The DNA pellets were dissolved in distilled water, fractionated using 2% agarose gels in TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA) and then transferred to a Zeta-Probe membrane (Bio-Rad, Richmond, CA) by capillary transfer in 10× SSC (1.5 mol/L sodium chloride, 0.15 mol/L sodium citrate). The membrane was exposed to an x-ray film at −70°C for 2–6 hours before development, and each of these experiments was carried out in triplicate.

Results

Two types of TUNEL-positive cells were seen in the posttraumatic rat brain. One type of TUNEL-positive cells (type I cells) appeared neuron-like but lacked morphological features of characteristic apoptosis such as cell shrinkage, nuclear condensation, or nuclear fragmentation. In fact, the triangular shape of neurons was frequently maintained. These cells demonstrated uneven TUNEL staining predominantly in the cytoplasm, but the nuclei were also stained (Figure 1, A–C). At the ultrastructural level, these cells displayed morphological features consistent with necrotic cell death (Figure 1C). The second type of TUNEL-positive cell (type II) was characterized by a round and shrunken morphology. The nuclei were round, condensed, and frequently fragmented into intensely TUNEL-positive nuclear fragments. Cytoplasmic TUNEL labeling, although usually not as strong as the labeled nuclear fragments, also was seen in the type II cells (Figure 1, D–F). Taken together, these features suggested that the type II cells were undergoing an apoptotic cell death. The cytoplasmic staining in both type I and II cells could be explained by leakage of low molecular weight DNA fragments into the cytoplasm as described previously.[26] Although DNA fragmentation was detected in the type I cells by TUNEL staining, the morphology of these cells was more characteristic for necrotic than for apoptotic cell death.

Both type I and II cells were detected in the injured cortex after both mild and moderate brain injury (Figure 2, A–D), but they were not seen in the analogous areas in the contralateral hemisphere (Figure 2, E and F) or in the brains of sham-injured animals (Figure 2, G and H). Most commonly, single TUNEL-

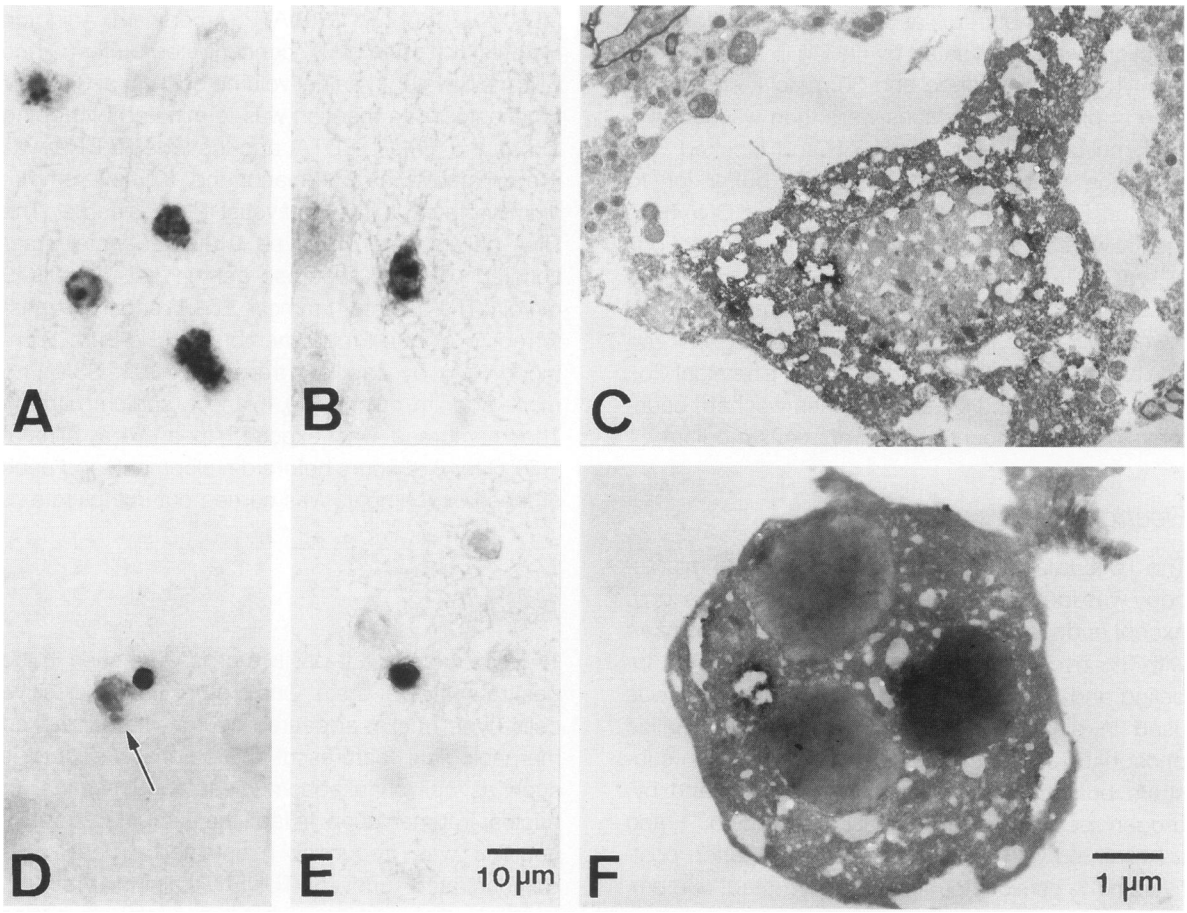


Figure 1. *Two types of TUNEL-positive cells (types I and II) were detected in rat brains after mild or moderate levels of LFP-TBI.* (**A–C**)*: Type I TUNEL-positive cells without cell shrinkage or nuclear condensation were seen at the light* (**A** *and* **B**) *and electron microscopic* (**C**) *levels. These cells showed strong cytoplasmic TUNEL staining, but only focal nuclear labeling.* (**D–F**)*: Type II TUNEL-positive cells were characterized by cell shrinkage, nuclear condensation, and fragmentation, also seen at the light* (**D** *and* **E**) *and electron microscopic* (**F**) *levels. These cells showed intense TUNEL staining in both the nucleus and the cytoplasm.* (**A**, **B**, **D**, *and* **E** *have the same magnification;* **C** *and* **F** *have the same magnification.*)

positive cells were interspersed among normal-looking TUNEL-negative neurons in cortex. Significantly more TUNEL-positive cells were seen in the subcortical white matter, dentate gyrus, and hippocampal CA3 region ipsilateral to the site of maximal cortical damage compared with the equivalent areas in the contralateral hemisphere of the injured rats or in the brains of the sham-treated animals. Interestingly, TUNEL-positive cells also were detectable bilaterally in the lateral geniculate body, which is relatively remote from the injured cortex.

To understand the temporal relationship between the induction of type I and II cells after FP brain injury, we quantitated the total number of TUNEL-positive cells as well as the number of type II cells in the cortex (Figure 3A). The number of type I and II cells in the injured cortex reached a peak at about 24 to 48 hours after mild or moderate trauma with a mean peak value of 107 and 119 cells/sector, respectively (Figure 3B). The number of type II cells in the cortex (Figure 2B) after mild injury reached a peak (mean peak value = 33 cells/sector) at about 24 hours after injury (Figure 3C). Accordingly, after mild severity lateral fluid percussion traumatic brain injury (LFP-TBI), about one-third of the TUNEL-positive cells were type II cells that appeared to undergo apoptotic cell death. The number of type II cells in the cortex subjected to moderate LFP-TBI remained relatively constant at different time points (range of mean = 4.0 to 9.67 cells/sector). Therefore, about 10% of the TUNEL-positive cells in the injured cortex appeared apoptotic (ie, type II cells) after brain injury of moderate severity (Figure 3C). In contrast, the number of apoptotic cells in the subcortical white matter was higher in animals subjected to moderate compared with mild injury (Figure 3D). Since type I and II cells may be eliminated very rapidly (ie, within a few hours) after showing evidence of TUNEL-positivity, our quantitative studies may have underestimated the abundance of apoptotic cells that result

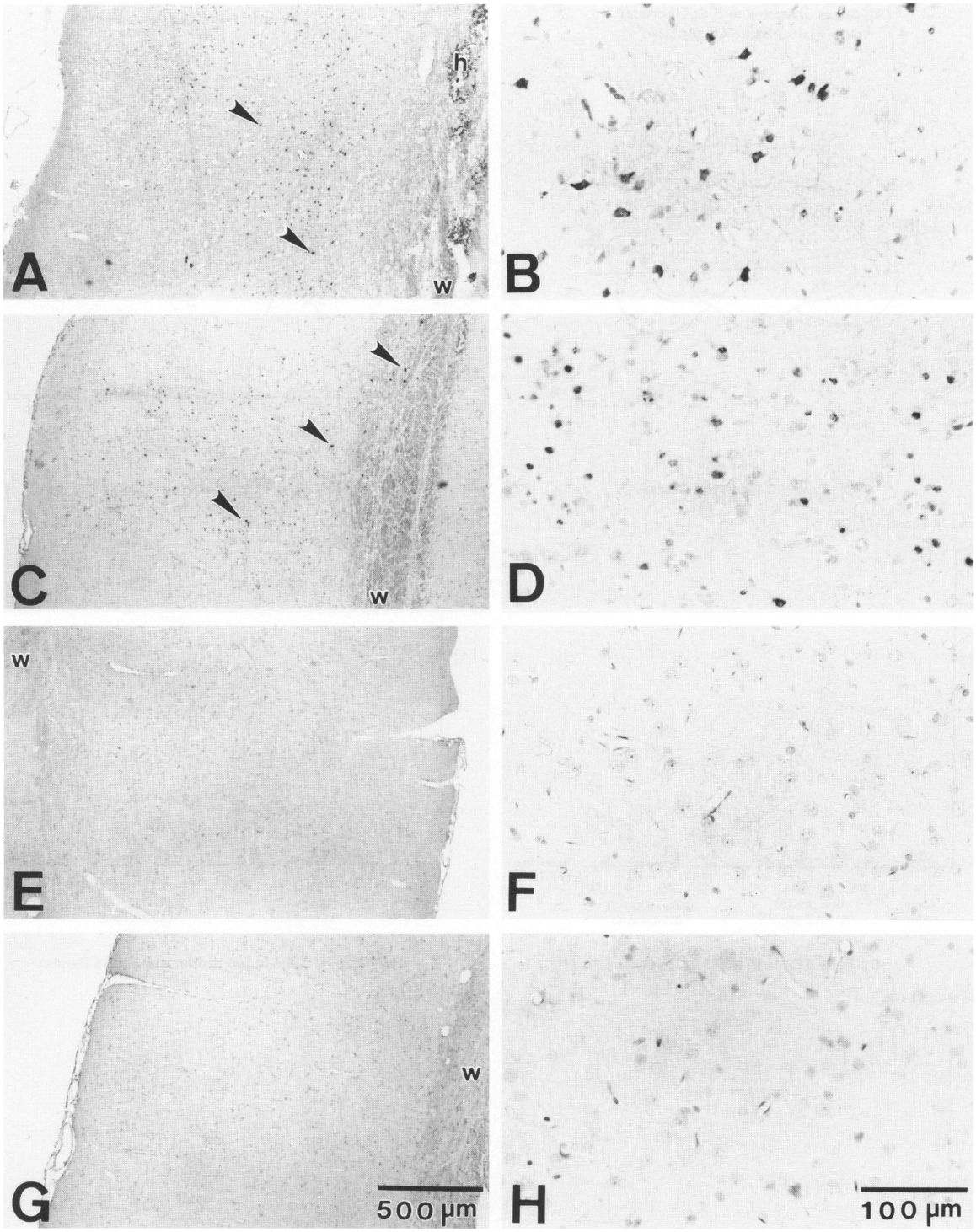
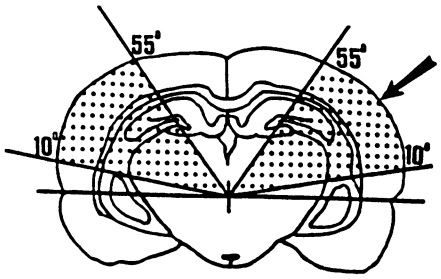


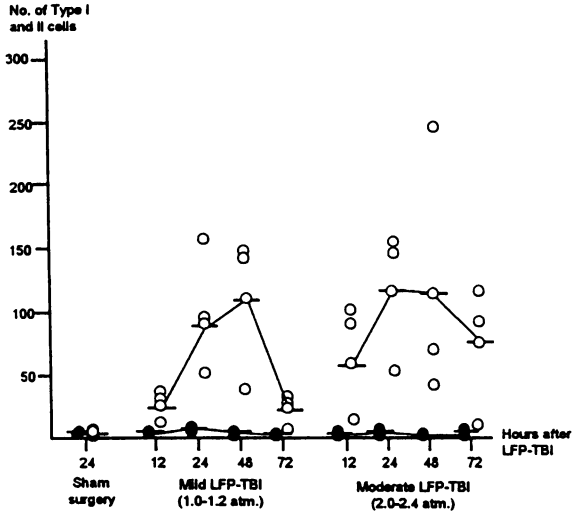
Figure 2. *TUNEL-positive cells* (arrows) *in the cortex 24 hours after LFP-TBI. TUNEL-positive cells were detectable in the cortex after both mild* (A *and* B) *and moderate* (C *and* D) *severity LFP-TBI. No TUNEL-positive cells were seen in the cortex contralateral to the injured site* (E *and* F) *or in the cortex after sham surgery* (G *and* H). *Abbreviations: w: white matter, h: hemorrhage.* (A, C, E, *and* G *have the same magnification;* B, D, F, *and* H *have the same magnification.*)

from LFP-TBI. The number of apoptotic cells detected in the dentate gyrus and the hippocampal CA3 region after mild brain injury peaked at 24 to 48 hours after injury (Figure 3, E and F). Notably, adjacent non-hippocampal and non-hilar neurons appeared unaffected. The number of apoptotic cells in

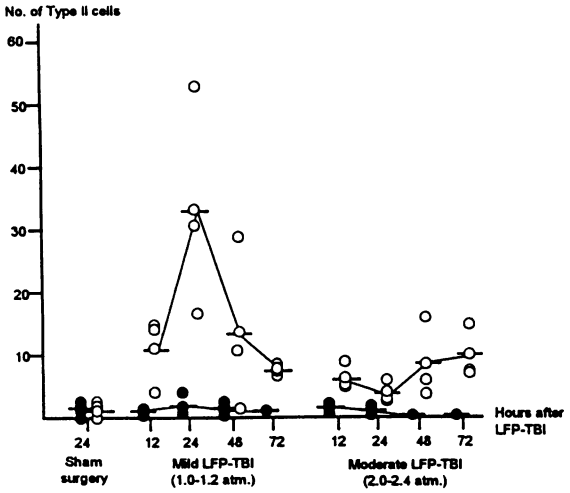
(A): Areas of Cortex and Sub-cortical White Matter being Quantitated



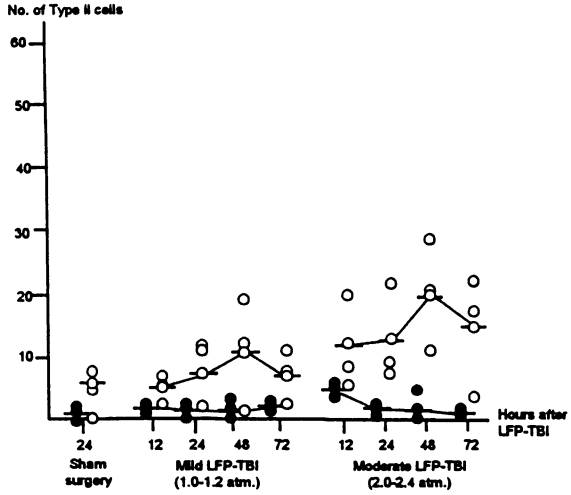
(B): Type I and II Cells in the Cortex



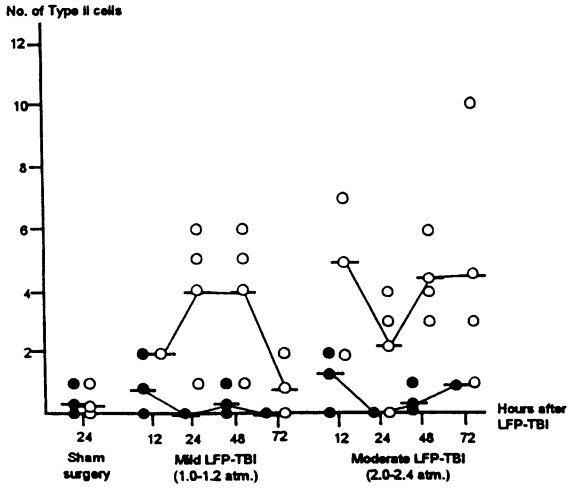
(C): Type II Cells in the Cortex



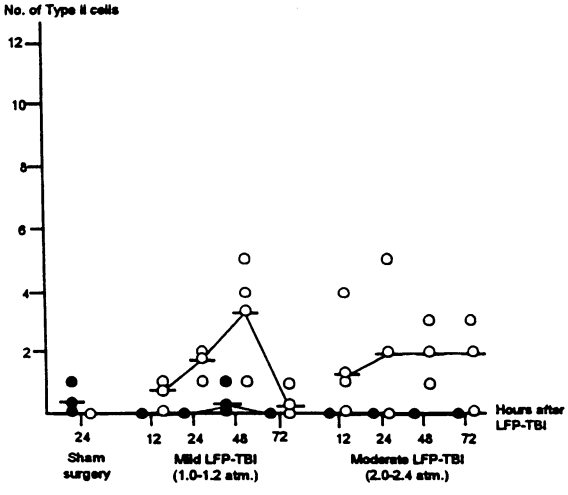
(D): Type II Cells in the Subcortical-White Matter



(E): Type II Cells in the Dentate Gyrus



(F): Type II Cells in the Hippocampal CA3 Region



the dentate gyrus and hippocampal CA3 regions after brain injury of moderate severity was comparable to the number seen in the same area after mild injury (Figure 3, E and F).

To further verify the presence of apoptotic mechanisms after brain trauma, we conducted studies to detect evidence of internucleosomal DNA fragmentation and randomized DNA fragmentation in the posttraumatic cortex and hippocampus by gel electrophoresis with a radioactive end-labeling method.[23] With this method, typical internucleosomal DNA fragmentation at intervals of 185 to 200 bp was revealed in the injured cortex and hippocampus after both mild and moderate injury. These fragments displayed a characteristic DNA "ladder" by gel electrophoresis. The injured cortex consistently showed more prominent DNA laddering than the corresponding hippocampi at the same level of injury. Also, in both the cortex and the hippocampus, there was a more prominent DNA ladder after moderate severity LFP-TBI compared to mild severity LFP-TBI. Random DNA fragmentation in the form of a DNA smear was not seen, and no DNA ladder was detectable in the cortex and hippocampus contralateral to the injured hemisphere (Figure 4).

Discussion

Under the criterion that TUNEL-positive cells are cells with extensive DNA damage and represent dying or dead cells, the time course and distribution of cell death after experimental TBI was evaluated.

In this study, two types of TUNEL-positive cells were detected. Type I TUNEL-positive cells displayed features consistent with necrotic cell death, whereas type II cells displayed features characteristic of apoptotic cell death. The demonstration of type II TUNEL-positive cells indicated that apoptosis represents one of the mechanisms of cell death after FP brain injury. Interestingly, apoptotic cell death was observed after both mild and moderate brain injury. It is not surprising to see TUNEL-positive staining in necrotic cells (ie, type I cells), because DNA in necrotic cells is expected to be extensively damaged. Because TUNEL-positive cells were observed even 72 hours after brain injury, delayed apoptotic cell death may be one mechanism underlying the protracted loss of neurons associated with progressive behavioral changes after brain injury. In addition, apoptotic cell death as detected by TUNEL-positive cells in the dentate gyrus and hippocampus (which are relatively remote from the injury site) indicates that these two areas are particularly vulnerable to apoptotic cell death after traumatic injury.

Although a large number of necrotic cells can be observed in the acute (24-hour) period after brain trauma, it is interesting that gel electrophoresis revealed only DNA laddering characteristic of apoptotic cell death at 24 hours after injury. The observation of DNA laddering in tissues clearly demonstrating morphological features of necrotic cell death has also been described previously.[27,28] Based upon our electron microscopy studies, we interpret the type I cells to be cells undergoing necrotic cell death and hypothesize that internucleosomal DNA fragmentation may represent the earliest stage of DNA fragmentation in necrotic cell death, with randomized DNA fragmentation representing the later stage of DNA damage. This suggestion is based largely on the observation that internucleosomal DNA represents DNA segments that are not coiled and therefore not protected by the nucleosome. During endonuclease digestion in apoptosis, it is not surprising, therefore, that these segments are initially cut by endonuclease. However, because necrosis may persist in tissue for a much longer time than apoptosis, the protection by nucleosomes is eventually compromised because of degradation of the histones. At this stage, randomized DNA fragmentation may begin. Moreover, necrosis, but not apoptosis, is accompanied by inflammation. The inflammatory cells may provide proteases that may digest the nucleosomes and facilitate randomized DNA fragmentation. In one recent study concerning the role of apoptotic cell death in human and experimental models of Huntington's disease, proteases have been shown to be necessary in the generation of DNA smear that has been reported in necrotic cells.[29] Therefore, we speculate that DNA laddering may be detectable at certain stages of both apoptotic and necrotic cell

Figure 3. *Quantification of TUNEL-positive cells in rat brain after mild severity LFP-TBI (1.0 to 1.2 atm, n = 12), moderate severity LFP-TBI (2.0–2.4 atm, n = 12) or sham surgery (n = 3). The site of LFP-TBI is indicated* (arrow). *The number of TUNEL-positive cells in the cortex and the subcortical white matter was quantitated in a sector* (A) *on both the injured and uninjured sides of the brain defined by two lines deviating 10 and 55 degrees from an imaginary line connecting the bilateral rhinal fissures* (dotted areas). *All TUNEL-positive cells in the dentate gyrus and hippocampal CA3 region also were counted.* (B) *Number of TUNEL-positive cells (types I and II) in the cortex.* (C–F): *Number of type II cells in the cortex, subcortical white matter, dentate gyrus, and hippocampal CA3 region, respectively. (Symbols: i, injured side; I, uninjured side; circles with a bar (connected), mean value).*

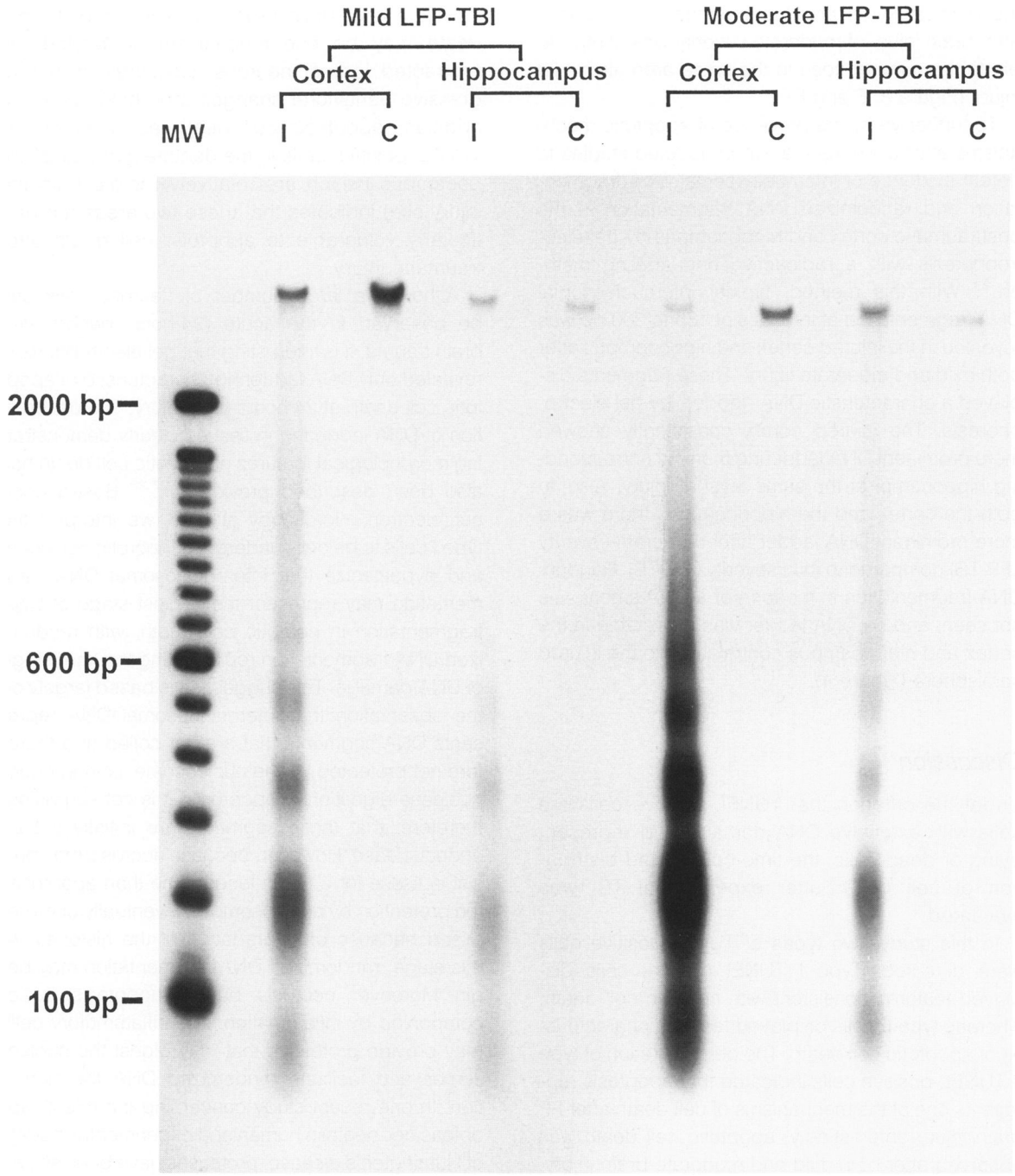


Figure 4. *Internucleosomal DNA fragmentation 24 hours after mild and moderate LFP-TBI. Gel electrophoresis revealed DNA fragmentation at the internucleosomal intervals of 185 to 200 bp in both the cortex and hippocampus from the injured (I) side but not contralateral (C) to the injured sides at both mild and moderate levels of injury. Molecular weight markers are shown on the left* (lane MW). *The positions of 100 bp, 600 bp, and 2000 bp are illustrated.*

death. Our data suggest that, in addition to the well described cascade of necrotic cell death, a temporal course of apoptotic cell death is initiated after brain trauma in selected brain regions. The activation of programmed cell death pathways may play a role in the pathophysiological response to traumatic brain injury.

Acknowledgments

We would like to thank Dr. Lucy B. Rorke for critical comments on the manuscript as well as Mr. Brian Perri and Ms. Justina M. Minda for their technical assistance.

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