

Young Investigator's Award: Induction of Apoptosis Following Traumatic Head Injury in Humans

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Abstract

Apoptosis or programmed cell death plays an important role in many developmental and pathological processes of the central nervous system. In head injury, apoptosis has been recently implicated in many studies on animal brain samples the phenomenon of apoptotic gene expression (bax and bcl-2).

Twenty specimens of contused brain tissue (temporal and frontal lobe) from 20 patients who underwent emergency craniotomy and removal of mass lesions were obtained from May to October 1997. The samples collected were immediately snap frozen in liquid nitrogen and stored at -80 °C. Immunohistochemical analysis was performed to detect the expression of bcl-2, bax and p53 using standard avidin-biotin complex second antibody conjugate methodology utilising commercially available primary and secondary antibodies.

The average age of cohort was 44.24 ± 22.17 years, the average Glasgow Coma Scale on admission was 9.19 ± 4.72, and the average duration from injury to collection of the sample was 20.62 ± 40.57 hours. There was documented hypoxia and hypotension seen in 5 of the 20 patients (25%). Significant levels of bax protein expression were noted in all samples, and p53 expression in 30% of samples. No bcl-2 expression was observed.

Our study showed that for the first time the strong expression of the pro-apoptotic gene (bax) and low levels of the anti-apoptotic gene (bcl-2), thus implicating the mechanism of apoptosis in brain injury following trauma. The use of agents to inhibit apoptosis may be beneficial in head injury patients.

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Introduction

The outlook for sufferers of severe head trauma is extremely poor. Some 20% of patients died before significant treatment can be administered, and nearly one quarter of patients end in a vegetative state with little potential for further progress. The remaining patients, while classified as having a favourable outcome, often suffer from long-term disabilities, physical impairment and neurological disabilities such as poor memory. Improved patient management, such as prompt surgical intervention to remove contused masses and aggressive management of intercranial pressure have helped to reduce the severity of injury in many cases, but large areas for the improvement of treatment still remain.

The extent of the final brain damage depends upon a number of factors, in addition to the causative nature of the injury. Often secondary insults, such as hypoxia and ischaemia can dramatically elevate the final level of damage sustained, and result in damage to the brain that is more global in nature than the primary injury would suggest. The presence of apoptotic cells as a response to brain injury from trauma has now been confirmed by several workers in experimental animal models and apoptotic cells have been detected in regions away from the site of trauma.^{1,2} While the mechanism of induction of apoptosis in response to brain trauma is not known, some evidence has suggested that apoptosis might be controlled by the bax/bcl-2 pathway. In particular, one

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report has suggested that the anti-apoptotic gene bcl-2 may be induced as a neuroprotective protein in response to experimental head trauma.³ Bax and bcl-2 are considered pivotal genes in the control of apoptosis, with the ratio of bax/bcl-2 determining cells fate. These two genes have been shown to normally exist as a heterodimer, and if bcl-2 is in excess, all of the available bax is bound, and the cell prevented from undergoing apoptosis. If bax is in excess, all available bcl-2 is bound and the break released and the cell dies. In this manner, bcl-2 is considered an inhibitor of apoptosis, while bax is a promoter of apoptosis.⁴ Interestingly, both of these genes are believed to be transcriptionally regulated by the p53 tumour suppressor gene, and activation of p53 has been shown to promote the transcription of bax, and repress the transcription of bcl-2.^{5,6}

Objective of Study

To investigate whether animal studies are germane to the events occurring in human head trauma patients, we sought to determine whether there was evidence of cellular apoptosis occurring in samples obtained during emergency craniotomy, as well as to examine whether genes of the bax/bcl2 pathway showed alterations in their level of expression.

Materials and Methods

Twenty patients were recruited into this study. All had undergone traumatic head injury and required subsequent emergency craniotomy. Trauma resulted from either a fall (9 cases), road traffic accident (4 cases), pedestrian accident (5 cases) or assault (2 cases). Patient assessment was carried out after 6 months whereby patients were assessed on the Glasgow Outcome Scale [Score 1 (Patient died) to 5 (return to daily living)]. Samples were collected at the time of surgery, snap frozen in liquid nitrogen and stored at -80°C until required. Immunoreactive p53, bcl-2 and bax were assayed by the labelled streptavidin-biotin method. Apoptotic cells were detected by the TUNEL methodology which detects the free 3'-hydroxyl groups generated during autonucleotitic digestion of DNA, one of the characteristic hallmarks of apoptosis.⁷ TUNEL analysis was undertaken with the Cell Death Kit (Boehringer Mannheim GmbH, Penzburg Germany) essentially as recommended by the manufacturer after fixing samples in a solution of 4% paraformaldehyde.

Results

TUNEL positive cells were detected in 95% (19/20) of patients (Fig. 1). In TUNEL positive samples, positive cells ranged from scattered single cells to samples where almost the whole section displayed apoptotic cells (Fig. 1). Apoptotic cells were noted in samples harvested as little as two hours after trauma and as late as 192 hours

Patient	TUNEL	p53	bcl2	bax	Outcome	Time (h)
1	+	-				2
2	+	-				3
3	+	-				
4	+/-	-		+/-		23
5	+/-	-		+		10
6	++	-		+/-		22
7	+++	+				
8	++	+		++		
9	++	-				12
10	++	-		+	4	12
11	+++	+		+	4	36
12	+	-			2	
13	++	-				
14		-				2
15	+	-				
16	++	-				192
17	+++	-		++	5	28
18	+++	+		++	4	4
19	+/-	+		+	5	4
20	++	+		++	3	26

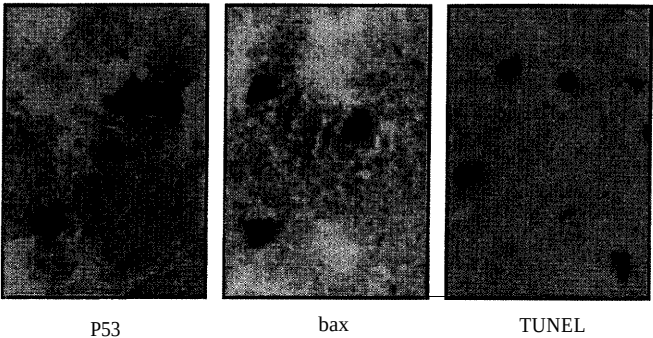


Fig. 1. Summary of results from 20 head trauma patients. Top: Results for both TUNEL analysis and immunohistochemistry were assessed as either negative (-) or on a scale of + (scattered positive single cells) to +++ (intense staining over majority of section). Outcome was assessed at 6 months post trauma on the Glasgow Outcome Scale. Time represents elapsed time between trauma and surgery in hours. Bottom: Representative samples for p53 (x100); bax (x100) and TUNEL analysis (x100).

after trauma. Maximal intensity appears to peak in samples collected between 4 and 36 hours after trauma (Fig. 1).

Immunohistochemistry for p53 was undertaken with the monoclonal antibody D07, (Dako, Carpinteria CA), a human specific antibody directed against an epitope between amino-acids 35 and 45 of the p53 tumour suppressor gene. This antibody is able to detect the p53 protein in both its active and denatured forms. Expression was detected in 30% (6/20) of patients (Fig. 1). In each case, staining was noted in single isolated cells and was restricted to nuclear expression. The low level of p53 positive cells detected here would be consistent with a transient increase in expression of p53 induced by the trauma. Immunoreactive p53 was detected in samples whose time from trauma to surgery ranged from 4 to 36 hours, with all of the cases operated on at 4 hours after

impact showing evidence of p53 immunoreactivity.

Immunohistochemistry for immunoreactive bcl-2 was undertaken with monoclonal antibody Ab-2 (Oncogene Research Products, Cambridge MA) an antibody directed against the human bcl-2 gene product. Immunoreactive bcl-2 was not detected at significant levels in any of the samples.

Immunoreactive bax was detected with monoclonal antibody AB-1 (Oncogene Research Products). Bax protein was detected in all samples examined, although 20% of samples displayed very low levels of reactivity. In many cases, bax staining was noted across a significant proportion of the section, in others, discrete, isolated areas of staining were noted (data not shown). The pattern of expression of bax gene product detected in this study is consistent with the known cellular location of the bax protein (Fig. 1). Immunoreactive bax was detected in samples harvested as early as two hours after trauma, and remained detectable in samples harvested as many as 192 hours after trauma. Maximal expression seemed to be detected at between 4 and 28 hours after trauma.

Discussion

It is clear from both animal model studies and the results presented here that cell death occurs in the brain after trauma, and a considerable body of evidence has accumulated which strongly support the contention that this cell death occurs by the regulated process of apoptosis. The process of programmed cell death, or apoptosis, was first described in 1972 by Kerr and colleagues.⁸ Since then, it has become evident that apoptosis is an essential part of growth and development, and the failure of appropriate apoptosis has been linked to many human disorders.⁷ As a mechanism of cell death, apoptosis is distinctive from necrosis in several key respects. In particular, apoptosis is tightly controlled and involves the co-ordinated action of many genes. In contrast necrosis is a passive event, requiring neither energy nor gene transcription.⁷

Apoptosis results in the cleavage of DNA at the nucleosomes, resulting in a characteristic DNA ladder when genomic DNA is extracted from apoptotic cells and analysed by gel electrophoresis. This cleavage is known to result in free 3'-hydroxyl groups on the DNA, and these free hydroxyl groups are now considered a characteristic marker of cellular apoptosis.⁷ While there

is some dispute over the ability of TUNEL assays to discriminate between apoptosis and necrosis: sufficient supporting evidence has been presented here (i.e. the induction of bax) to confidently ascribe positive staining to apoptosis rather than necrosis. The fact that cell death in the brains of head trauma patients is occurring by a process of apoptosis rather than necrosis has obvious clinical implications. Particularly pertinent in this respect is the work of Sinson and colleagues¹⁰ who have shown in rats that both the extent of apoptosis and the degree of subsequent learning deficit can be significantly reduced by continual infusion of nerve growth factor after experimentally induced trauma. Such novel therapeutic approaches, based upon a rational understanding of the mechanism of cell death induced by trauma, when applied to human patients may well serve to alleviate the long-term consequences of severe head trauma.

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