Cell Death Mechanisms Following Traumatic Brain Injury

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Neuronal and glial cell death and traumatic axonal injury contribute to the overall pathology of traumatic brain injury (TBI) in both humans and animals. In both head-injured humans and following experimental brain injury, dying neural cells exhibit either an apoptotic or a necrotic morphology. Apoptotic and necrotic neurons have been identified within contusions in the acute post-traumatic period, and in regions remote from the site of impact in the days and weeks after trauma, while degenerating oligodendrocytes and astrocytes have been observed within injured white matter tracts. We review and compare the regional and temporal patterns of apoptotic and necrotic cell death following TBI and the possible mechanisms underlying trauma-induced cell death. While excitatory amino acids, increases in intracellular calcium and free radicals can all cause cells to undergo apoptosis, in vitro studies have determined that neural cells can undergo apoptosis via many other pathways. It is generally accepted that a shift in the balance between pro- and anti-apoptotic protein factors towards the expression of proteins that promote death may be one mechanism underlying apoptotic cell death. The effect of TBI on cellular expression of survival promoting-proteins such as Bcl-2, Bcl-x_L, and extracellular signal-regulated kinases, and death-inducing proteins such as Bax, c-Jun N-terminal kinase, tumor-suppressor gene, p53, and the calpain and caspase families of proteases are reviewed. In light of pharmacologic strategies that have been devised to reduce the extent of apoptotic cell death in animal models of TBI, our review also considers whether apoptosis may serve a protective role in the injured brain. Together, these observations suggest that cell death mechanisms may be representative of a continuum between apoptotic and necrotic pathways.

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INTRODUCTION

That death of both neurons and oligodendrocytes may contribute to the overall pathology of clinical and experimental brain trauma is becoming increasingly evident. While neuronal death is associated with focal injuries, death of oligodendrocytes may be a hallmark of diffuse brain trauma. A variety of studies (1, 39, 54, 92) have suggested that focal contusions in the gray matter and diffuse injuries to axons in the white matter may be a consequence of the biomechanics of the impact, ie, focal injuries arise due to contact forces to the head, while diffuse injuries result from non-contact, rotational forces to the brain (64). While multiple neurochemical processes and intracellular pathways appear to be invoked following a traumatic insult to the brain, it is unclear whether cell death in CNS injury follows the same pattern of initiation-commitment-execution stages that has been extensively characterized in models of developmental neuronal death (80). We review here the state of the current literature describing the patterns of cell death following traumatic brain injury (TBI) in humans and animals, and the possible pathways that lead to apoptotic and/or necrotic cell death following traumatic injury to the CNS. As more information regarding the pathologic changes and the underlying cellular and molecular phenomena associated with TBI becomes available, it appears that TBI is a complex neurodegenerative disease. Thus, the strategies necessary to design a successful therapeutic regimen may need to be carefully planned and evaluated. While apoptotic pathway(s) may provide reasonable targets for therapeutic interventions, it may be simplistic to develop an approach that is based on a very limited number of targets.

REGIONAL CELL DEATH PATTERNS FOLLOWING TBI

TBI in humans results in neuronal loss in the cortex, hippocampus, cerebellum and thalamus (1, 54, 92), and these patterns of cell death have been replicated in several animal models (38, 87). The depth and extent of contusions in various parts of the injured human brain have been quantitatively evaluated (1), and bilateral loss of hippocampal neurons has been observed in 85% of fatal head injury cases as early as 48 hours following the traumatic event (54). In the early post-traumatic period (hours to days), injured neurons in contusions appear swollen, but over time (days or weeks), they become shrunken and eosinophilic, with pyknosis of the nuclei (12). In experimental models of brain injury in the rat, neuronal degeneration is evident in the injured cortex and hippocampus in the opening minutes to hours following impact (25, 45, 110). Electron microscopic analysis of these degenerating neurons revealed a general swollen appearance, with swollen mitochondria, vacuolated cytoplasm and pyknotic nuclei (25, 110), suggestive of necrosis. Interestingly, observations of a time-dependent increase in the volume of the cortical lesion, and the presence of degenerating (dystrophic) neurons in the chronic post-traumatic period, have led to the suggestion that delayed or chronic neuronal degeneration may be a significant component of post-traumatic pathology (8, 21, 25, 45). Injury to the white matter is characterized by the widespread distribution of injured axons, which, in the acute post-traumatic stage, appear as swollen fibers containing accumulated cytoskeletal proteins (39). Over time, these swollen axons eventually undergo complete axotomy reminiscent of Wallerian degeneration (86), a process that is associated with death of oligodendrocytes (5). Traumatic oligodendrocyte death may also be induced by the reactive microglia and astrocytes present in the white matter (23, 39, 102).

Apoptosis, the morphological manifestation of programmed cell death (PCD),

is typically accompanied by internucleosomal DNA strand breaks. Gavrieli and colleagues (36) developed the terminal deoxynucleotidyl transferase-mediated dUTP nick end-lableing (TUNEL) technique which has been extensively utilized to identify these strand breaks in situ. Neurons and oligodendrocytes containing nuclear DNA fragments and exhibiting apoptotic morphology have been identified in humans following acute neurologic insults such as stroke, and brain and spinal cord trauma (18, 28, 62, 103). The contribution of apoptosis to the neuropathology of experimental brain trauma has been established using a variety of techniques such as TUNEL to detect DNA strand breaks in situ, the bis-benzimide dye to demonstrate nuclear DNA condensation, and electron microscopy to demonstrate apopototic morphology (17, 33, 76, 91, 121). Apoptotic neurons have been observed in the cortex and hippocampus in both brain-injured rats and mice (17, 20, 33, 52, 76). Interestingly, Conti and coworkers (22) have reported that there was a biphasic increase (at 24 hours and one week post-injury) in the number of apoptotic cells in the cortex, and a delayed appearance of apoptotic cells in the injured thalamus at one week post-injury. In addition to neurons, oligodendrocytes and astrocytes appear to undergo apoptosis following experimental TBI (22, 76). That neural cell apoptosis contributes to the pathology of TBI was confirmed by the presence of TUNEL(+) neurons and oligodendrocytes in human head-injured tissue (18, 103). In a recent series of studies, Graham and coworkers (101, 118) have demonstrated that apoptotic, TUNEL(+) cells are present in significant numbers predominantly in white matter tracts of traumatically-injured human brains up to 12 months post-injury.

CALCIUM AND POST-TRAUMATIC CELL DEATH

Over a decade ago, it was suggested that calcium-mediated mechanisms were the "final common pathway" leading to cell death following CNS injury (125). Increases in intracellular calcium have been demonstrated following experimental TBI (32, 78, 100). One immediate consequence of increased intracellular calcium concentrations is the activation of the calcium-activated neutral proteases, calpains, which have been implicated in the pathogenesis of traumatic brain injury (49, 63, 75, 94). Calpain activation leads to the limited proteolysis of a number of intracellular proteins including cytoskeletal proteins, particularly in regions that exhibit cell loss (50). Although calpain activation may lead to either necrosis or apoptosis, in vitro studies have suggested that in the setting of CNS injury, activation of calpain may be predominantly associated with necrotic cell death (116).

Evaluation of cell death mechanisms in in vitro systems has led to the hypothesis that low intracellular calcium concentration ([Ca]i) may selectively initiate apoptotic pathways, whereas high [Ca]i induces necrosis (44). In contrast, cell death following traumatic or ischemic insults in vivo may involve a more complicated scenario, involving altered anti- and pro-cell death signaling pathways. For example, the death-inducing activity of members of the Bcl-2 family (Bax, Bad, Bid, Bcl-x_S) appears to be in a dynamic equilibrium with their survival-promoting cognates (Bcl-2, Bcl-x_L) (65). As a result of these shifts in intracellular levels of Bcl-2 family proteins, the death-inducing cysteine proteases, caspases, are activated (111). Disruption of the balance between mitogen activated protein kinase (MAPK)-mediated intracellular signaling pathways may also control the fate of the cell—activation of c-Jun N terminal kinase (JNK) or p38MAPK may lead to cell death while extracellular-signal regulated kinase (ERK1/2) and Akt kinase are critical regulators of cell survival (119). In addition, oxidative DNA damage may lead to the induction of transcription factors such as p53, which, in turn, may mediate cell survival and/or death (31). Moreover, the cross-talk between various signaling pathways within cells underscores the complex nature of death-inducing stimuli.

CELL DEATH REGULATION BY THE BCL-2 FAMILY OF PROTEINS

Although originally characterized as genes that are associated with developmental cell death, recent evidence suggests that the Bcl-2 family of genes may participate in pathologic apoptotic and necrotic cell death (9). Neurotoxin- or ischemia-mediated apoptotic death was preceded by increased Bax mRNA and protein, and decreased expression of Bcl-2 in cells that are destined to die (40, 41), while an increase in Bcl-2 immunoreactivity was observed in neurons, glia and endothelial cells that survived focal ischemic injury (14). Similarly, increased expression of Bcl-2 has been observed in neurons that survive the traumatic insult both in the rat and in brain-injured humans (17, 18), while Bax was observed to translocate to the nucleus of apoptotic cells following experimental brain injury (52). Alternatively, recent studies have suggested that decreases in intracellular Bcl-2 immunoreactivity, with little to no change in Bax proteins, in injured brain regions may precede cell death following experimental brain trauma (88, 90). Transgenic mice overexpressing the human Bcl-2 protein exhibited significantly less neuronal loss in the injured cortex and hippocampus following experimental TBI, lending support to the idea that Bcl-2 may participate in the neuronal cell death following TBI (71, 89). A pro-apoptotic member of the Bcl-2 family, Bid, has also been implicated in trauma-induced cell death in vivo—proteolysis of Bid preceding its translocation to the mitochondria has been demonstrated in the injured cortex (34). Bcl-2 family members may likely control cell death by regulating the release of cytochrome c from mitochondria (124), which has been observed in both neuronal cell bodies and axons following experimental brain trauma (11, 108). Once in the cytosol, cytochrome c aids in activation of the apoptosis-promoting cysteine family of proteases, caspases.

CELL DEATH AND CASPASE ACTIVATION

Activation of caspases has been associated with neuronal and oligodendroglial cell death resulting from multiple kinds of stimuli such as growth factor deprivation, hypoxia, free radical generation, ionizing radiation, and ischemia (43, 72, 74, 83). Currently, 14 members of the mammalian caspase family have been identified, separated into 2 categories: the "activator" caspases such as caspase-8 and -9, and the "executioner" caspases, such as caspase-2, -3, -6 and -7 (111). Activation of caspase-9, which has been demonstrated in vivo following experimental cerebral ischemia (55) and traumatic spinal cord injury (105), has been suggested to occur prior to and mediate the activation of caspase-3 (111). Activation of caspase-3 has been reported in

injured cerebral cortex in the hours to days following experimental (7, 19, 53, 84, 108, 121) and human (18) brain injury. Recent evidence has implicated both caspases-8 and -9 as putative initiator caspases for trauma-induced caspase-3 activation, albeit in a regionally distinct manner (6, 53, 122). Activation of caspases-8 and -9 appear to precede caspase-3 activation in the injured cortex (6, 53, 122), while caspase-3 activation in the hippocampus and thalamus appears to be preceded predominantly by activation of caspase-9 (53).

That caspase-mediated cell death may participate in the pathobiology of CNS injury was further substantiated in studies employing both caspase inhibitors and transgenic mice overexpressing mutant caspases demonstrating that reduced caspase activity led to neuroprotection in models of stroke (29, 35). Similarly, Yakovlev and coworkers demonstrated that post-traumatic apoptotic cell death and neurological deficits were reduced by administration of the caspase inhibitor, z-DEVD-fmk, following lateral fluid-percussion brain injury in the rat (121). More recently, Clark and coworkers reported that while post-traumatic administration of z-DEVD-fmk attenuated neuronal apoptosis and reduced the extent of cortical injury following experimental brain trauma, brain-injured, z-DEVD-fmk-treated rats were as impaired in motor function as their vehicle-treated counterparts (19).

The current hypothesis regarding the mechanism of caspase-mediated cell death proposes that caspases cleave multiple proteins, the sum of which leads to cell death (111). For instance, caspases may cleave anti-apoptotic regulators such as the inhibitor of the nuclease responsible for DNA fragmentation, as well as cytoskeletal proteins (eg, spectrin and actin) resulting in the disassembly of the dying cell (58, 59). The characteristic internucleosomal DNA fragmentation that is observed in apoptotic human cells has been suggested to be mediated via a specific endonuclease, the 40-kDa DNA fragmentation factor (DFF40) (61). DFF40 is present in the cytosol as a heterodimer with a 45-kDa subunit, DFF45, and upon cleavage of DFF45 by activated caspase-3, translocates to the nucleus as the active nuclease (61). Cleavage of rat DFF45 homolog and subsequent translocation of DFF40-like protein to the nucleus has been demonstrated in the cortex and hippocampus of rats following TBI (127). Moreover, the post-traumatic cleavage of the DFF45-like protein was attenuated in brain-injured animals treated with the caspase-3 inhibitor, z-DEVD-fmk (19). Caspase-3-mediated cleavage of the cytoskeletal protein, actin, has been observed in the injured cortex in the acute post-traumatic period, and in the injured thalamus at 3 weeks post-injury (4). More recently, active caspase-3 has also been implicated in the pathogenesis of traumatic axonal injury with the observation of the specific formation of caspase-3-cleaved amyloid β-peptide fragment in injured axons (107).

CELL DEATH REGULATION BY MAP KINASES

Both JNK and ERK1/2 are known regulators of cell survival/death in a number of neural and non-neural systems in vitro. Phosphorylation (activation) of JNK signaling has been associated with neuronal cell death and activation of ERK1/2 kinase linked to cell survival (119). In vivo, systemic administration of kainic acid leads to an acute and sustained decrease in phospho-ERK1/2 levels, and a concomitant increase in phospho-JNK in apoptotic neurons within the cortex and hippocampus (66). Delayed neuronal death following global cerebral ischemia is preceded by a sustained increase in activated JNK (82), while increased ERK1/2 signaling is associated with neuroprotection in a model of ischemic preconditioning (99). Activated JNK was evident in both apoptotic neurons as well as in apoptotic oligodendrocytes following compressive spinal cord injury (70). Inhibition of JNK directly or blocking upstream activators of JNK has been reported to attenuate apoptotic cell death in vitro (119) and in vivo (96). Recently, gene-targeted disruption of the brain isoform of JNK, JNK3, resulted in mice that were resistant to kainic acid-mediated hippocampal cell death and exhibited reduced seizure activity and mortality (123).

Recent studies in models of CNS injury in vivo appear to suggest an alternate mechanism. Both ischemic (2) and traumatic (24, 68, 81) brain injuries appear to activate ERK1/2 in injured brain regions. Phospho-ERK1/2 appears to colocalize with both neuronal (24, 68) and astrocytic (81) markers in the injured cortex and hippocampus. In contrast, JNK activation was not observed in the injured cortex (68), and only transiently in both vulnerable and invulnerable cells in the hippocampus (81), suggestive of a lack of correlation between trauma-induced cell death and JNK activation. Whether ERK activation in injured neurons is associated with cell death or is an attempt by injured cells to maintain normal function is yet to be determined. Pre-injury treatment of animals with a specific inhibitor of ERK phosphorylation, PD98059, has been observed to decrease ERK activation and the extent of cell death after injury (68), but appears to exacerbate cognitive and motor deficits in brain-injured animals (24).

DNA DAMAGE AND POST-TRAUMATIC CELL DEATH

Although controlled DNA fragmentation (ie, breakage of both DNA strands) is one biochemical hallmark associated with apoptosis (as shown above), it has also been reported that single and/or double DNA strand breaks may trigger apoptotic cell death (98, 114). While activation of endonucleases can result in double-stranded breaks in DNA, single stranded breaks typically occur due to oxidative damage (98). Damage to the DNA activates intracellular pathways that lead to either growth arrest and apoptosis or repair and elimination of damaged DNA(31), a choice that is made based on the cell type, extent of damage and/or environment. One major component of the DNA damage response is the induction and upregulation of the tumor suppresser gene, p53, also termed the "guardian of the genome" (31). Induction of p53 mRNA has been associated with neuronal damage following excitotoxic and ischemic brain injuries (16, 48, 95). Following experimental brain injury, increased mRNA and protein for p53 were observed in regions that exhibit neuronal apoptosis and in neurons that were TUNEL(+) (52, 73). Interestingly, despite reports that p53-deficient mice are resistant to both excitotoxic and ischemic injuries (69), p53-deficient mice exhibited as much cortical and hippocampal damage as their wild-type counterparts following traumatic brain injury (unpublished observations). Because wild-type p53 is a transcription factor for genes such as wild-type p53 activated fragment (WAF1/p21)(3), the pro-apoptotic

factor, Bax (67), and the growth arrest and DNA damage-inducible gene, GADD45 (126), the consequences of p53 induction are many. While WAF1 and GADD45 can cause cell cycle arrest and facilitate DNA repair and eventual cell survival, Bax can induce cell death.

Oxidatively damaged DNA may be repaired by the base-excision repair (BER) pathway and recent reports suggest that the DNA damage that occurs following ischemic brain injury may be subjected to BER (98). Indirect evidence for the activation of BER following ischemia and trauma arises from the observations of increased activity of poly (ADP-ribose) polymerase (PARP)(30, 56). Because PARP uses nicotinamide adenine dinucleotide as its substrate, thereby depleting cellular stores of energy, some studies have suggested that PARP activation following CNS injury may be detrimental, and post-injury inhibition of PARP activity appeared to prevent cell death (27, 57, 117). Furthermore, a decrease in nuclear levels of the endonuclease associated with BER following global cerebral ischemia suggests that neuronal apoptosis occurs as a result of a failure of DNA repair processes (51).

CELL DEATH MECHANISMS: A CONTINUUM BETWEEN APOPTOSIS AND NECROSIS?

Until recently, it was widely believed that the morphology (mitochondrial swelling, nuclear pyknosis, disruption of the plasma membrane) of dying cells indicated that cell death following acute neurologic insults such as hypoxia-ischemia, seizures or trauma was predominantly via necrosis. Although a number of studies have implicated a role for the apoptosis in the mature CNS following ischemic or traumatic injury, these types of injuries to the adult CNS may not necessarily result in classical apoptosis, such as that described during development of the nervous system (93). Instead, the appearance of morphologic features of both necrosis and apoptosis in the same neural cell, and the presence of only some of the characteristics of developmental apoptosis, has led to the possibility that a continuum between apoptosis and necrosis exists (77, 85). It is equally likely that the nature and/or intensity of the insult may regulate whether a complex cell such as a neuron undergoes apoptosis or necrosis. Although moderate-severe ischemia induces primarily necrotic cell death in the cortex (13), neuronal apoptosis is the predominant pattern of cell death following mild focal ischemia (26). Experimental TBI of moderate severity results in both necrosis and apoptosis, with necrosis contributing to a greater extent than apoptosis to the number of all dying cells (22, 76). In contrast, apoptotic cells appear to contribute to a similar extent as necrosis within the injured cortex and subcortical white matter following mild lateral fluid-percussion brain injury in rats (88). Alternatively, it has been reported that activation of N-methyl-D-aspartate subtype of the glutamate receptor may lead to necrosis, while non-NMDA receptor activation may underlie apoptotic neuronal death (85). The presence of a continuum would suggest that intracellular pathways that lead to apoptosis and necrosis may also not be mutually exclusive. For example, although calpains may mediate necrosis and caspase-3 is only activated in apoptotic cells (116), calpain activation may also lead to apoptosis (106).

Based on the dependence of apoptosis on energy in the form of ATP, some researchers have suggested that intracellular ATP concentrations may regulate whether a cell undergoes necrosis or apoptosis (42, 112). Thus, as long as ATP is present within the injured cell, apoptotic pathways may be initiated, and once ATP is depleted (as a result of damage to the mitochondria), the injured cell may shift towards necrosis. This hypothesis may, in part, explain why neurons dying as a result of a pathologic stimulus may exhibit features of both apoptosis and necrosis, ie, the apoptotic features may represent the temporal extent to which apoptotic pathways were active. Mitochondrial dysfunction associated with decreases in ATP has been documented following experimental TBI (109, 115, 120), although more recent data from the lateral fluid-percussion brain injury model suggest that TBI-induced decreases in ATP levels may not be sufficient to inhibit apoptosis (60). It has also been reported that reversal of trauma-induced mitochondrial damage by cyclosporin A treatment inhibits traumatic cortical cell loss (97), and axonal injury (10, 79).

BENEFITS OF APOPTOTIC CELL DEATH IN THE TRAUMATICALLY-INJURED BRAIN

In the developing brain of both vertebrates and invertebrates, apoptotic neuronal death is a means by which the neurons that have not formed meaningful synaptic connections are removed. In addition, the occurrence of apoptosis during development also serves to promote signal plasticity (80). One of the hallmarks of apoptotic cell death is that there is minimal activation of the immune system as a result of cell loss, and thus, surrounding cells can remain relatively unaffected. Thus, it remains conceivable that neuronal apoptosis following an ischemic or traumatic insult may represent a protective response by the brain, ie, a mechanism by which the brain is able to remove injured/damaged cells and only minimally affect the remaining brain tissue. In this regard, induction of apoptosis using staurosporine resulted in a larger cortical lesion in rats subjected to focal cerebral ischemia (15). Clark and coworkers (19) demonstrated that although caspase-3 inhibition reduced the number of apoptotic neurons in the cortex following experimental TBI, caspase inhibitor-treated animals were as impaired in motor function as their vehicle-treated counterparts. Similarly, the cortical and hippocampal damage following TBI in transgenic mice overexpressing the anti-apoptotic protein, Bcl-2 was significantly reduced compared to that in the wild-type mice, but motor and cognitive deficits in brain-injured Bcl-2 transgenic mice were not alleviated (71, 89).

Outside the mature CNS, apoptosis is a typical mechanism by which cells within the immune and circulatory systems are removed, to be replaced by newly-born, healthy cells. In addition, lymphocytes in autoimmune spinal disease and activated microglia in injured peripheral nerves are eliminated over time via apoptosis (37, 113). Inflammation is a significant component of the early pathologic response after TBI, characterized by the infiltration of macrophages and neutrophils and the activation of resident CNS microglia (46, 47, 104). The numbers of these inflammatory cells peak within 2 to 3 days following TBI, and over the following days or weeks return to control (baseline) levels. However, it is unclear whether some of the apoptotic cells in the traumatically-injured rat brain

may represent dead or dying macrophages and/or microglia.

CONCLUSIONS

Taken together, the concepts outlined in this review highlight the heterogeneity of the pathologic and molecular responses to TBI. While great strides are being taken in the understanding of the regional and temporal patterns of the cellular and molecular responses in the traumatically injured brain, the ever-increasing amount of information underscores the complexity of the disease. Though the quest to identify potential targets to inhibit neuronal cell death and to develop strategies for the treatment of the clinical condition must continue, this path must be tempered with a rational approach to treatment design.

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