Upregulation of the Fas Receptor Death-Inducing Signaling Complex after Traumatic Brain Injury in Mice and Humans

Jianhua Qiu,[1]* Michael J. Whalen,[1]* Pedro Lowenstein,[2] Gary Fiskum,[3] Brenda Fahy,[3] Ribal Darwish,[3] Bizhan Aarabi,[4] Junying Yuan,[5] and Michael A. Moskowitz[1]

[1]Neuroscience Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129, [2]Gene Therapeutics Research Institute, Cedars-Sinai Medical Center, and Department of Medicine, University of California at Los Angeles, Los Angeles, California 90048, Departments of [3]Anesthesiology and [4]Neurosurgery, University of Maryland School of Medicine, Baltimore, Maryland 21201, and [5]Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

Recent studies have implicated Fas in the pathogenesis of inflammatory, ischemic, and traumatic brain injury (TBI); however, a direct link between Fas activation and caspase-mediated cell death has not been established in injured brain. We detected Fas–Fas ligand binding and assembly of death-inducing signaling complexes (DISCs) [Fas, Fas-associated protein with death domain, and procaspase-8 or procaspase-10; receptor interacting protein (RIP)–RIP-associated interleukin-1β converting enzyme and CED-3 homolog-1/Ced 3 homologous protein with a death domain–procaspase-2] by immunoprecipitation and immunoblotting within mouse parietal cortex after controlled cortical impact. At the time of DISC assembly, procaspase-8 was cleaved and the cleavage product appeared at 48 hr in terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling-positive neurons. Cleavage of caspase-8 was accompanied by caspase-3 processing detected at 48 hr by immunohistochemistry, and by caspase-specific cleavage of poly(ADP-ribose) polymerase at 12 hr. Fas pathways were also stimulated by TBI in human brain, because Fas expression plus Fas–procaspase-8 interaction were robust in contused cortical tissue samples surgically removed between 2 and 30 hr after injury. To address whether Fas functions as a death receptor in brain cells, cultured embryonic day 17 cortical neurons were transfected with an adenoviral vector containing the gene encoding Fas ligand. After 48 hr in culture, Fas ligand expression and Fas–procaspase-8 DISC assembly increased, and by 72 hr, cell death was pronounced. Cell death was decreased by ~50% after pan-caspase inhibition (Z-Val-ALa-Asp(Ome)-fluoromethylketone). These data suggest that Fas-associated DISCs assemble in neurons overexpressing Fas ligand as well as within mouse and human contused brain after TBI. Therefore, Fas may function as a death receptor after brain injury.

Key words: traumatic brain injury; Fas; death-inducing signaling complex; caspases; human; adenoviral vectors

Traumatic brain injury (TBI) causes acute as well as delayed, progressive cell death mediated in part by excess extracellular glutamate and derangements in intracellular calcium (Faden et al., 1989). Cell death is also mediated by caspases, a family of cysteine proteases that cleave cellular proteins specifically at aspartate residues (for review, see Raghupathi et al., 2000). Caspase-1 and caspase-3 are activated in contused rodent brain (Yakovlev et al., 1997; Beer et al., 2000b; Clark et al., 2000) and in contused brain from patients with severe TBI (Clark et al., 1999). In addition, pharmacological inhibition or genetic deletion of caspases reduces cell death and improves functional outcome after experimental TBI (Yakovlev et al., 1997; Raghupathi et al., 1998; Fink et al., 1999; Nakamura et al., 1999; Clark et al., 2000). Based on these studies, it seems likely that activation of caspases after TBI constitutes an important cell-death mechanism. However, the mechanisms initiating cell death after TBI are not well understood.

Fas receptor is a prototype member of the tumor necrosis factor/nerve growth factor receptor superfamily of death receptors identified in brain (Nagata, 1999). After Fas ligation, Fas receptor associates with Fas-associated protein with death domain (FADD) and initiator procaspases such as procaspase-8, procaspase-10, or procaspase-2 to form a "death inducing signaling complex" (DISC). Recruitment of initiator procaspases to this complex results in their autoactivation (Boldin et al., 1995; Chinnaiyan et al., 1995; Kischkel et al., 1995; Medema et al., 1997). Caspase-8 may process and activate effector caspases, such as caspase-3, directly. Alternatively, caspase-8 may activate caspase-3 indirectly by cleaving and activating the cytosolic bid that promotes cytochrome *c* release, apoptosome formation, and activation of caspase-9 and caspase-3. Thus, DISC formation is a key upstream event that links activation of death receptors with initiation of caspase-mediated cell death.

Several lines of evidence suggest that death receptors participate in neuronal death after traumatic and ischemic CNS injury (Cheema et al., 1999; Raoul et al., 1999, 2000; Felderhoff-Mueser et al., 2000; Rosenbaum et al., 2000; Martin-Villalba et al., 2001). However, a direct link between death-receptor activation, DISC

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*J.Q. and M.J.W. contributed equally to this work.

Correspondence should be addressed to Dr. Michael A. Moskowitz, Massachusetts General Hospital, 149 13th Street, Room 6403, Charlestown, MA 02129. E-mail: Moskowitz@helix.mgh.harvard.edu.

formation, and activation of caspases after brain injury remains to be established.

The aim of this study was to test the hypothesis that TBI induces Fas death receptors and DISC assembly and promotes the initiation of caspase cascades that lead to cell death. Using a mouse controlled cortical impact (CCI) model and contused brain samples removed from patients with severe TBI, we show that death-receptor activation and DISC formation are upregulated and temporally associated with activation of initiator and effector caspases in injured brain. Furthermore, we show that overexpression of Fas ligand (FasL) in cultured neurons using transfection with an adenoviral vector induces DISC assembly and cell death that is attenuated by a broad-spectrum caspase inhibitor. The data indicate that death receptors may constitute an important initiating mechanism of cell death after TBI.

MATERIALS AND METHODS

Mouse controlled cortical impact model. The mouse CCI model (refers specifically to the murine experimental TBI model) was used as described previously (Whalen et al., 1999a,b) with minor modifications. The trauma protocol was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and complied with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were anesthetized with 2% isoflurane, N_2O and O_2 (2:1) using a nose cone. Mice were positioned in a stereotaxic frame and a brain temperature probe (Physitemp Corp., Clifton, NJ) was inserted through a burr hole into the left frontal cortex. Body temperature was monitored with a rectal probe and maintained at 36–38°C with a heating pad. A 5 mm craniotomy was made using a portable drill and a 5 mm trephine over the left parietotemporal cortex and the bone flap was removed. Brain temperature was maintained at 36°C for 1 min. Mice were then subjected to CCI using a pneumatic cylinder with a 3 mm flat-tip impounder, a velocity of 6 m/sec, and a depth of 0.6 mm. The bone flap was immediately replaced and the scalp was sutured closed. Anesthesia was discontinued and the mice were allowed to recover in room air until able to ambulate (~5 min) and were then returned to their cages.

Animal protocols. For light microscopy studies [terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labeling (TUNEL), hematoxylin and eosin (H&E), and immunohistochemical staining for leukocytes], mice subjected to CCI were killed by decapitation under isoflurane anesthesia at 0, 3, 6, 24, or 48 hr after injury. Brains were removed, frozen in isopentane at −40 to −50°C, and stored at −80°C. Within 1 week the brains were sectioned on a cryostat (10 μm) and stained.

For immunofluorescence histochemistry, mice were killed at 0, 6, 12, 24, or 48 hr after CCI. Cryostat brain sections were prepared as described above and stored at −80°C before staining.

Because immunoprecipitation and Western blots require the use of different homogenization buffers, separate groups of mice were used for each. For immunoprecipitation studies, mice were decapitated under isoflurane anesthesia at 0, 3, 6, 12, or 24 hr after CCI. Contused cortical tissue was carefully removed, immediately frozen in liquid nitrogen, and stored at −80°C. For Western blotting, mice were killed as described above at 0, 0.5, 3, 6, 12, 24, 48, or 72 hr after CCI. Brain tissue was frozen in liquid nitrogen and stored at −80°C.

For all experiments, three to four animals were used for each time point.

Antibodies. Anti-Fas antibodies (M-20 and C-20), anti-FasL antibodies (N-20 and C178), anti-caspase-8 antibodies (H-134 and D-8), anti-caspase-10 antibody (H-131), and anti-receptor interacting protein (RIP)-associated interleukin-1β converting enzyme and CED-3 homolog-1 (ICH)/Ced 3 homologous protein with a death domain (RAIDD) (FL-199) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FADD polyclonal antibody was purchased from Chemicon International Inc. (Temecula, CA). Monoclonal antibody against Fas ligand/CD95L (clone 33), Fas/CD95/Apo-1 (clone 13), and RIP (clone 38) were obtained from Transduction Laboratories (Lexington, KY). Anti-caspase-10/a monoclonal antibody was supplied by R&D Systems (Minneapolis, MN). Mouse anti-caspase-2 (ICH-1) (clone G310-1248) antibody was purchased from PharMingen International Inc. (San Diego, CA). Anti-poly(ADP-ribose) polymerase (PARP) (C-2–10) monoclonal antibody was obtained from Alexis Biochemicals (San Diego, CA). Anti-neutrophil antibodies were purchased from Serotec (Raleigh, NC). The specificity of the antisera to cleaved (active) fragments of caspase-8 (SK440) and caspase-3 (SK398) has been characterized previously (Velier et al., 1999; Matsushita et al., 2000). Specificity of the commercially available antibodies was confirmed by detection of a major band of the appropriate molecular weight on Western blot.

Immunohistochemistry and TUNEL. Coronal brain sections placed on poly-L-lysine-coated slides were fixed in 100% ethanol at −20°C for 10 min and then washed in PBS, pH 7.4, containing 0.1% Triton X-100 (PBST). Sections were blocked for 1 hr in PBST containing 5% normal goat serum and then incubated for 1–3 d with rabbit polyclonal primary antibodies at 4°C. Sections were washed in PBST and incubated with goat anti-rabbit IgG–Cy3 conjugate (Jackson ImmunoResearch, West Grove, PA) for 60 min. After washing in PBST, sections were incubated with mouse monoclonal anti-mouse NeuN (1:300) for 60 min and then reacted with goat anti-mouse IgG–bodipy (1:300; Molecular Probes, Eugene, OR) for 30 min. For TUNEL staining, sections were incubated with TdT buffer (in mM: 30 Tris, 140 sodium cacodylate, 1 cobalt chloride, pH 7.2) containing TdT (0.5 U/ml) and biotin-16–deoxyUTP (dUTP) (0.04 mol/l) (all reagents from Boehringer Mannheim, Indianapolis, IN) for 1 hr at 37°C. The reaction was terminated by washing in PBS. Biotin-16–dUTP incorporated into tissue was reacted with streptavidin–Cy5 (1:1000, Jackson ImmunoResearch) for 5 min for immunofluorescence microscopy. After washing in PBS, sections were dehydrated in an ascending ethanol series, immersed in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Triple-labeled sections were analyzed on a Leica (Nussloch, Germany) DMRB/Bio-Rad (Hercules, CA) MRC 1024 krypton–argon laser-scanning confocal microscope. Excitation/emission filters were 488/522 nm for bodipy, 568/585 nm for Cy3, and 650/670 nm for Cy5, respectively. Double-labeled sections were analyzed on a Nikon (Tokyo, Japan) Eclipse T300 fluorescence microscope. Negative controls included incubation with rabbit serum instead of primary antibodies or omission of secondary antibodies, biotin-16–dUTP, or Cy5. Specificity controls also included varying the order of reaction with the different antibodies and TUNEL.

Immunoblotting. Brain tissue or cultured neurons were homogenized on ice in buffer A (10 mM HEPES buffer, pH 7.6, 42 mM KCl, 5 mM $MgCl_2$, 1% SDS, 1 mM phenylmethylsulfonylfluoride, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1.5 μM pepstatin, 2 μM leupeptin, and 0.7 μM aprotinin). The lysate was cleared by centrifugation at 20,800 × *g* for 30 min at 4°C. The protein content of the supernatant was assayed (Bio-Rad), and proteins were size fractionated on 10% or 10–20% SDS-polyacrylamide gels and blotted onto a Hybond nitrocellulose membrane (Amersham Biosciences, Arlington Heights, IL) overnight. The blot was blocked for 1 hr in 5% milk in PBST and then incubated overnight at 4°C in primary antibodies diluted according to the recommendations of the manufacturer. Membranes were washed in PBST and 1% milk and then incubated for 1 hr with the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature. Proteins of interest were detected using the enhanced chemiluminescence (ECL) Western blotting detection system kit (Amersham Biosciences, Buckinghamshire, UK) and Hyperfilm (Amersham Biosciences, Oakville, Ontario, Canada) and analyzed by densitometry using an M4 imaging system (Imaging Research, Inc., St. Catherines, Ontario, Canada).

Immunoprecipitation. Physical interaction between proteins was determined by immunoprecipitation analysis of (1) cortical tissue from mice killed at various times after CCI; (2) cortical tissue removed from patients with severe TBI, refractory seizure disorders, or postmortem; or (3) cultured neurons after Fas ligand overexpression. Brain tissue was lysed, homogenized, and pelleted by centrifugation in buffer containing 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride, 50 mM NaF, 0.7 μM aprotinin, and 10% glycerol. The supernatants were precleared by incubation with protein G-agarose and normal rabbit IgG for 2 hr at 4°C and were incubated with 2 μg of anti-Fas antibody (M-20), anti-caspase-8 antibody (H-134), and anti-RAIDD (FL-199) or anti-Fas ligand (C-178) antibody overnight at 4°C. Lysates were then incubated with protein G-agarose for 2 hr at 4°C. The immunoprecipitates were washed three times with buffer containing 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 0.5% deoxycorticosterone, 1% NP-40, and 62.5 mM NaCl and subsequently dissolved in denaturing sample buffer.

The immunoprecipitates were separated by 10–20% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking with 5% skim milk in TBS with 0.05% Tween 20 (Sigma,

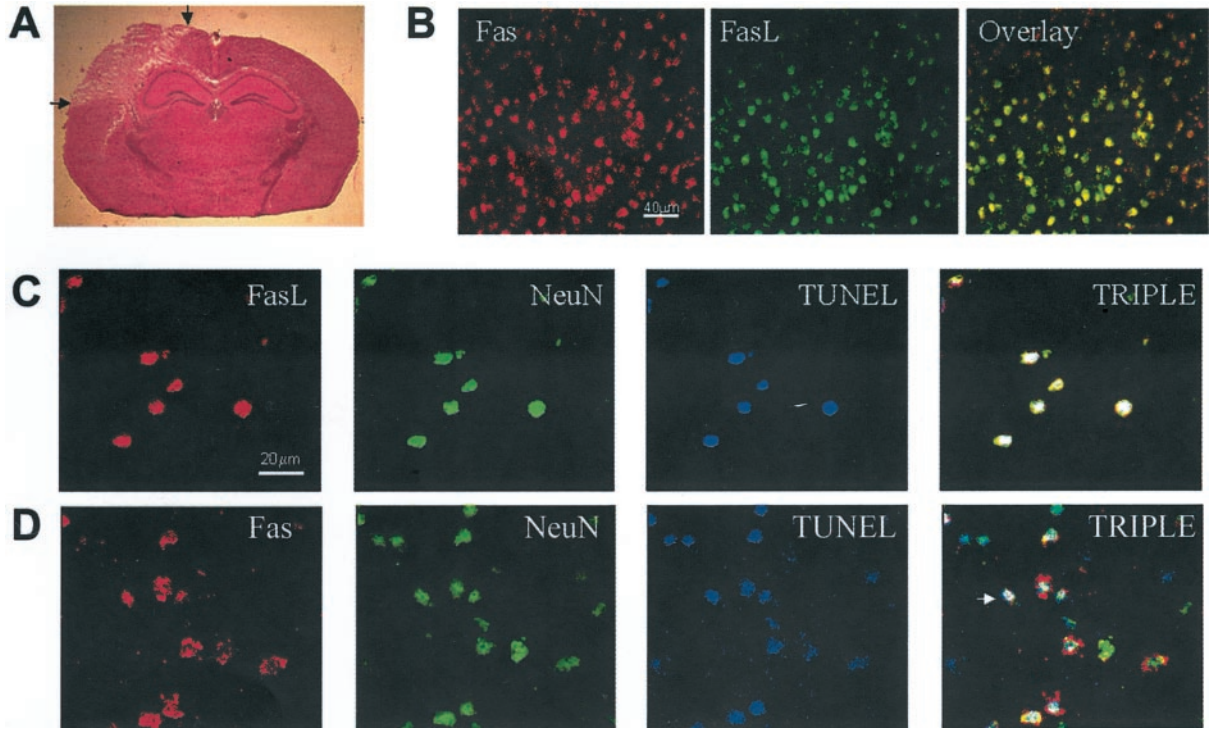

Figure 1. Anatomic localization of cortical contusion and colocalization of Fas ligand and Fas in cortical neurons after CCI. Mice subjected to CCI were killed after 6 hr, and cryostat brain sections were processed by H&E staining (*A*) or by immunohistochemistry (*B–D*). *A*, Anatomic location in the parietal cortex of the lesion produced by CCI. *B*, Colocalization of Fas and Fas ligand in normal mouse cortical neurons. Fas ligand was labeled with mouse monoclonal antibody (clone 33) and goat anti-mouse IgG–Cy2 (*green*). Fas receptor was labeled with rabbit polyclonal antibodies (M-20) and goat anti-rabbit IgG–Cy3 (*red*). Brain sections were analyzed by fluorescence microscopy. Double-labeled cells appear *yellow*. *C, D*, Colocalization of Fas and Fas ligand with TUNEL-positive neurons. Fas ligand (*C*) and Fas receptor (*D*) were labeled with rabbit polyclonal antibodies N-20 and M-20, respectively, and with Cy3 (*red*). Neurons were detected using mouse monoclonal anti-mouse NeuN and visualized with goat anti-mouse bodipy (*green*); TUNEL-positive cells were labeled with biotin-16–dUTP–streptavidin–Cy5 (*blue*). TUNEL-positive neurons colabeled with anti-Fas ligand or anti-Fas stain *white* (*arrow* in *D*).

St. Louis, MO), the membranes were incubated with anti-caspase-8 (D-2), anti-Fas (clone-13), or anti-Fas ligand (clone-33) antibody followed by reaction with the appropriate horseradish peroxidase-conjugated secondary antibody. Protein was detected using the ECL system and Hyperfilm (both from Amersham) and semiquantitated by densitometry with the M4 imaging system (Imaging Research).

Analysis of human brain for Fas upregulation and Fas–procaspase-8 interaction. Contused brain samples surgically removed from patients between 2 and 30 hr after severe TBI were analyzed by Western blot and immunoprecipitation (above) for expression of Fas receptor and for interaction between Fas and procaspase-8. The patients were admitted to the R. Adams Cowley Shock Trauma Center of the University of Maryland Medical Center and the use of tissue samples was approved by the Institutional Review Board. Cerebral contusion, present in all patients, was documented by computed tomography scan. Control brain samples were taken within 24 hr postmortem from adults who died of non-CNS related causes ($n = 1$) and from cerebral cortex excised from patients with intractable seizure disorders ($n = 5$). Brain tissue was stored at −80°C until use.

Primary neuronal culture and Fas ligand overexpression. Primary cultures of cerebral cortical neurons were prepared from embryonic day 16 (E16) or E17 C57BL/6 mice (Charles River Laboratories, Wilmington, MA). Cells were isolated using trypsin and cultivated in neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, and 25 μM glutamate (Invitrogen, San Diego, CA). Cells were seeded at a density of 1×10^6 cells/well in six well plates coated with poly-D-lysine and then incubated in a humidified atmosphere of 5% CO_2 at 37°C. On day 3, the cultures were incubated with 10 μM cytosine arabinoside for 24 hr to suppress the growth of glial cells. One-half of the medium in each well was changed every 4 d. At day 4, glutamate was withdrawn from the medium. Neurons cultured for 1 week were used in all experiments.

Cultured cortical neurons were incubated with recombinant adenovirus (rAd)–cytomegalovirus (CMV)–Fas ligand (Morelli et al., 1999) (40 pfu/cell) for up to 3 d at 37°C. As a control, neurons were also incubated with rAd–CMV–lacZ at 40 pfu/cell. Dead cells were determined by exclusion of trypan blue and Hoechst staining. Cells were collected by scraping at day 2 after transfection for analysis by immunoprecipitation and Western blot.

To determine the sensitivity of cell death to a caspase inhibitor, cultured neurons were transfected with adenovirus containing Fas ligand in the presence of 500 μM Z-Val-ALa-Asp(Ome)-fluoromethylketone (ZVAD-fmk) in 0.1% dimethylsulfoxide (DMSO) or vehicle alone. At 72 hr after transfection, dead cells were counted using trypan blue staining. A total of three wells with three areas per well and 100 cells/area were analyzed for each condition.

Statistical analyses. *t* tests were used to determine differences in densitometry measurements between groups and differences in neuronal survival *in vitro*. $p < 0.05$ was considered significant.

RESULTS

Histopathology of contused cortex after CCI in mice

The spatiotemporal distribution of cell death in the mouse model was similar to that reported by others after CCI in mice (Smith et al., 1995; Whalen et al., 1999a) and in rats (Colicos et al., 1996). The anatomic location of the contusion produced by CCI is shown in Figure 1*A*. Within 3–4 hr of CCI, the cortical contusion was characterized by edema, hemorrhage, and hypereosinophilic staining of cells with the morphologic appearance of neurons. These "red neurons" were observed in ipsilateral but not contralateral cortex and hippocampus and were distributed among normal-appearing cells in the impact zone. By 6–12 hr, the number of red neurons in injured cortex was markedly increased

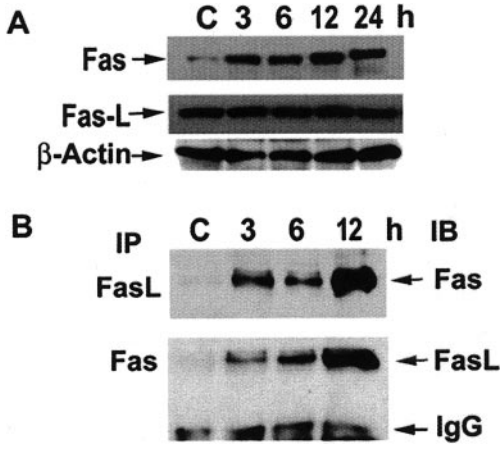

Figure 2. Expression of Fas and Fas ligand (*A*) or Fas–Fas ligand interaction (*B*) after CCI in mice over time [in hours (*h*)]. Contused or normal (uninjured control; *C*) cortex was subjected to Western blot analysis using polyclonal anti-Fas (M-20) or anti-Fas ligand (N-20). Fas and Fas ligand were constitutively expressed in uninjured cortex. Expression of Fas receptor was increased after CCI, whereas there was no change in Fas ligand expression. Bands corresponding to β-actin were of equal intensity in all *lanes*, suggesting equal protein loading. *B*, Fas–FasL interaction is increased after CCI. Fas ligand or Fas receptor was immunoprecipitated from cortical supernatant homogenates with the corresponding polyclonal antibodies and subjected to SDS-PAGE. Western blots were probed with monoclonal anti-Fas or anti-Fas ligand, respectively. A time-dependent increase in Fas–Fas ligand interaction was found. Robust interaction between Fas and Fas ligand was observed regardless of whether anti-Fas or anti-Fas ligand was used to immunoprecipitate. Bands corresponding to light-chain IgG were of equal intensity in all *lanes*, suggesting equal antibody loading. *IP*, Immunoprecipitation antibody; *IB*, immunoblotting antibody.

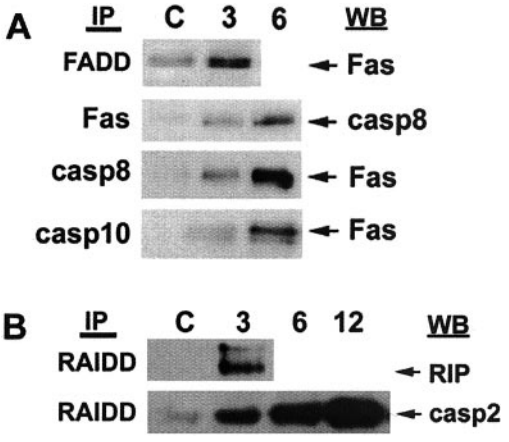

Figure 3. DISC assembly after CCI. *A*, Cortical samples were analyzed by immunoprecipitation using anti-FADD, anti-Fas, anti-caspase-8, or anti-caspase-10 antibodies, and were subjected to SDS-PAGE. Immunoblots were probed with antibodies against caspase-8 (H-134) or Fas receptor (clone 13). Interaction between Fas receptor and FADD, procaspase-8, and procaspase-10 was apparent after CCI. Bands corresponding to light-chain IgG were of equal intensity in all *lanes*, suggesting equal protein loading (data not shown). *B*, Traumatic brain injury promotes Fas–RIP–RAIDD–caspase-2 interaction. Mice were subjected to CCI and killed at the indicated times (in hours). Brain tissue from contused cortex was then analyzed by immunoprecipitation using anti-RAIDD. Immunoprecipitants were probed on an immunoblot using anti-RIP and anti-caspase-2 antibodies. Interaction of RIP–RAIDD–caspase-2 increased at 3 hr after injury. Bands corresponding to light-chain IgG were of equal intensity in all *lanes*, suggesting equal antibody loading (data not shown). *C*, Control (normal) mouse cortex; *IP*, immunoprecipitation antibody; *WB*, immunoblotting antibody.

both in the center as well as at the margins of the contusion. A similar distribution of cells with DNA damage, as assessed by TUNEL staining, was observed at both early (3–4 hr) and later times after injury. Neutrophils were first detected within contused cortex at 24 hr after CCI, and increased at 24–48 hr in five of six animals. Most neutrophils were found scattered throughout the contusion, but some were distributed in the perivascular space as well. Cell death assessed by H&E and TUNEL staining appeared maximal at 24–48 hr and was largely complete after 72 hr. At 7 d, a cavitary lesion was present.

Increased expression of Fas and Fas ligand early after CCI

We first examined whether Fas and Fas ligand were expressed in normal mouse brain and upregulated after CCI. Both Fas and Fas ligand colocalized with nearly all NeuN-positive cells (data not shown), and both Fas and Fas ligand colocalized with each other in nearly all cells with neuronal morphology in normal mouse cortex (Fig. 1*B*). Fas and Fas ligand were detected in the cytosol of TUNEL-positive neurons at 6 hr after CCI (Fig. 1*C,D*). During the first 12 hr after CCI, only a minor fraction of the total number of cells expressing Fas and Fas ligand was NeuN-negative.

Constitutive expression of Fas and FasL was detected in homogenates of mouse parietal cortex (Fig. 2*A*). As reported previously after CCI in rats (Beer et al., 2000a), Fas expression increased as early as 30 min (data not shown) and was sustained for up to 72 hr after injury. Fas expression was increased fourfold versus controls at 3 hr after CCI and at all subsequent times examined ($p < 0.05$). FasL expression remained at control levels and did not increase when examined up to 72 hr in our model (Fig. 2*A*; data not shown).

Fas–Fas ligand binding is increased after CCI

Binding of specific ligands to death receptors induces receptor trimerization and activation. We reasoned that if TBI caused Fas activation, then we should detect a time-dependent increase in Fas–Fas ligand interaction. Fas–Fas ligand interaction was detected in injured cortex early after CCI (Fig. 2*B*). β-actin was not detected in the same immunoprecipitant (data not shown), thereby suggesting that our result was most likely attributable to specific Fas–Fas ligand binding.

DISC is formed by Fas, FADD, and procaspase-8 or procaspase-10 after CCI

Fas ligand engagement promotes association of the cytosolic domain of Fas with a cytosolic adapter FADD, which in turn recruits initiator procaspases. To examine whether DISC formation accompanies TBI, we used immunoprecipitation and Western blots to detect interaction between Fas, FADD, and procaspase-8 or procaspase-10. Figure 3*A* shows marked, robust upregulation of Fas–FADD–procaspase-8 and Fas–procaspase-10 interaction as early as 3 hr after CCI. Interaction between Fas and procaspase-8 was either not detected at all or only weakly detected in normal mouse cortex. Hence, Fas receptor contributes to rapid and robust DISC assembly early after CCI. Because we observed red blood cells in contused brain at 3–6 hr after CCI, we assessed the possible contribution of activated blood cells to DISC formation in the postmortem brain. First, arterialized blood was withdrawn by cardiac puncture from a naive mouse or a mouse 6 hr after CCI. Five microliters of fresh whole blood was then injected into the cortex (left hemisphere) of a freshly perfused (saline) normal mouse brain. DISC assembly was assessed as described above. In both cases, the results of Fas–procaspase-8 coim-

munoprecipitation did not differ from controls (data not shown). These data suggest that blood contaminants do not contribute significantly to DISC formation in the traumatically injured brain. They do not rule out a contribution from blood at later times when leukocyte populations in the brain are more common.

RIP–RAIDD–caspase-2 interaction after CCI in mice

Fas signaling may also activate the initiator procaspase-2 by interacting with RIP, a serine/threonine kinase that associates with RAIDD, a FADD-like cytosolic adapter protein. The Fas–RIP–RAIDD complex can form a DISC by recruiting procaspase-2. In addition, RIP has been shown to mediate necrosis in non-neuronal cells, through mechanisms that remain unknown (Holler et al., 2000). We demonstrated a marked increase in RIP–RAIDD and RAIDD–procaspase-2 interaction at 3–12 hr after CCI (Fig. 3*B*). Little or no interaction between RIP–RAIDD or RAIDD–procaspase-2 was detected in normal brain.

Activation of caspase-8 and caspase-3 after CCI in mice

Because DISC formation does not imply significant downstream caspase activation, we looked for processing of caspase-8 and caspase-3 in injured brain. The p18 fragment of processed caspase-8 was detected in mouse brain as early as 3 hr after TBI but was absent in uninjured brain (Fig. 4*A*), whereas the proform was detected in normal brain homogenate. Processed caspase-8 and caspase-3 were also detected in TUNEL-positive neurons by immunohistochemistry at 48 hr after CCI (Fig. 4*B*). We did not detect cleaved caspase-8 and caspase-3 in neurons at early time points using immunohistochemistry (data not shown). Nevertheless, these data suggest that activation of initiator and effector caspases are events that are downstream after DISC formation.

To determine whether caspase activity is present after TBI, we examined contused brain for the 85 kDa cleavage fragment of PARP. The p85 PARP cleavage fragment, specific for cleavage by caspases, was detected in contused cortex at 12–24 hr after CCI but not in normal cortex (Fig. 4*C*).

Neuronal cell death is induced by overexpression of Fas ligand *in vitro*

To establish that Fas is a functional death receptor in neurons, we overexpressed Fas ligand in cultured neurons and assessed Fas–procaspase-8 coimmunoprecipitation as well as whether Fas ligand overexpression induced caspase-mediated cell death. Cells transfected with adenoviral vectors containing the gene encoding Fas ligand overexpressed Fas ligand protein twofold compared with cells transfected with control vector ($p < 0.05$) (Fig. 5*A*). Moreover, specific Fas and procaspase-8 protein–protein interactions were present in these neurons but not in controls (Fig. 5*B*). Between 48 and 72 hr, Fas ligand-expressing neurons showed morphologic features of apoptosis by Hoechst staining (data not shown), and by 72 hr, 80% of neurons were dead ($p < 0.05$ vs control). When cultured neurons overexpressing Fas ligand were treated with a pan-caspase inhibitor, ZVAD-fmk (500 μM), cell death was decreased to ~50% of control levels ($p < 0.05$) (Fig. 5*C*). These data indicate that Fas functions as a death receptor in cultured neurons, and that caspases constitute one mechanism mediating neuronal death induced by Fas ligand.

Fas forms a DISC after TBI in human brain

Based on our results in a mouse model, we hypothesized that Fas contributes to DISC formation after human TBI. Five male patients (16–75 years of age) with severe TBI (initial Glasgow coma scale scores of 3–12) suffered head injury after motor vehicle accidents ($n = 2$), falls ($n = 2$), or assault ($n = 1$). Compared with controls, Fas receptor was increased twofold ($p < 0.05$ vs control) (Fig. 6*A,B*) and Fas–procaspase-8 coimmunoprecipitation was increased ~75% ($p < 0.02$ vs control) (Fig. 6*C,D*) in brains from TBI patients. Notably, Fas–procaspase-8 interaction was only modestly increased in human brain samples containing large amounts of blood versus those containing lesser amounts (as judged from the amount of heme in the homogenate), arguing against a relationship between blood content and Fas–procaspase-8 DISC assembly (data not shown). Thus, Fas is associated with DISC formation during the pathogenesis of human TBI.

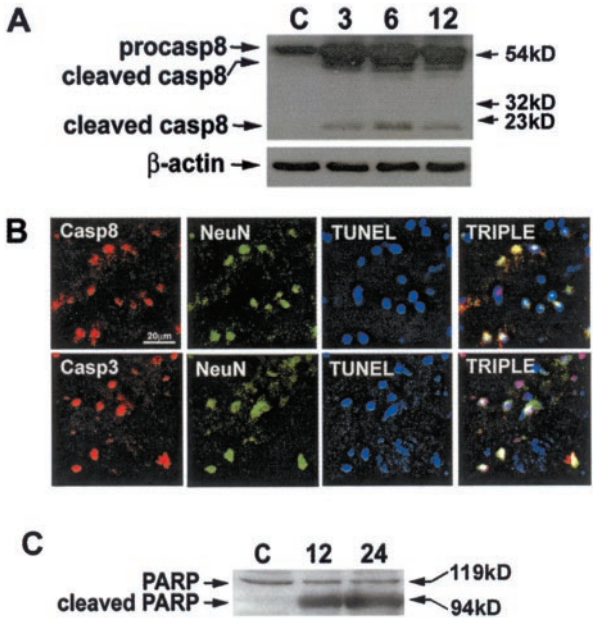

Figure 4. Caspase activation after traumatic brain injury in mice. *A*, Cleavage of caspase-8 early after CCI. Cortical homogenates from contused mouse cortex were analyzed by Western blot using an antibody (H-138) that reacts with the proform (p55) and active fragment (p18) of caspase-8. Cleavage of p55 to p18, as well as another caspase-8 cleavage product (p43) was observed by 3 hr after injury. β-actin was also probed to confirm equal protein loading. *C*, Control (normal) mouse cortex. *B*, Immunohistochemical detection of activated caspase-8 and caspase-3 in TUNEL-positive cortical neurons 48 hr after CCI. Cryostat brain sections (10 μm) taken from the center of the contusion were processed by immunohistochemistry. Caspase-8 (p18) or caspase-3 (p20) was detected with rabbit polyclonal antisera SK440 or SK398, respectively, and Cy3 (*red*). Neurons were identified using monoclonal anti-mouse NeuN and visualized with goat anti-mouse bodipy (*green*). TUNEL-positive cells were labeled with biotin-16–dUTP–streptavidin–Cy5 (*blue*). TUNEL-positive neurons colabeled with p18 or p20 appear *white*. At 48 hr after CCI, many of the TUNEL-positive neurons in the injured cortex colabeled with p18 and p20. *C*, PARP cleavage in brain after CCI. Cortical homogenates were analyzed by Western blot at the times indicated. The p85 PARP fragment, specific for caspase-mediated proteolysis of PARP, was robust at 12 and 24 hr after CCI. Bands corresponding to β-actin were of equal intensity in all *lanes*, suggesting equal protein loading.

DISCUSSION

Fas-induced cell killing has been implicated as a major mechanism of neuronal death in the developing brain (Cheema et al., 1999; Raoul et al., 1999; Felderhoff-Mueser et al., 2000), after cerebral ischemia (Martin-Villalba et al., 1999; Felderhoff-Mueser et al., 2000; Rosenbaum et al., 2000; Martin-Villalba et al., 2001; Northington et al., 2001), and after TBI (Beer et al.,

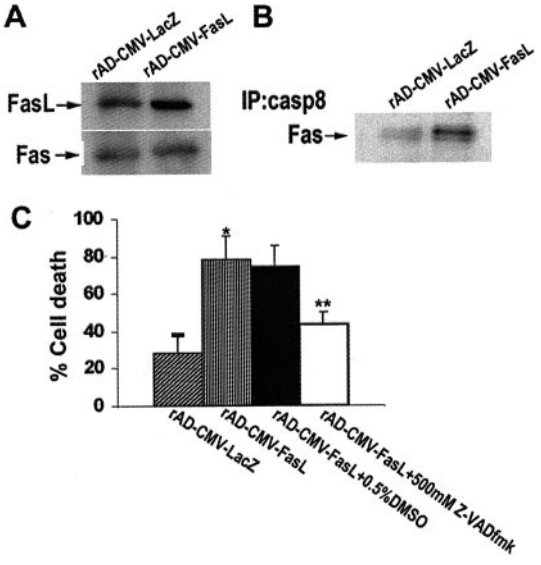

Figure 5. Fas ligand overexpression in enriched cultured cortical neurons. *A*, Expression of Fas ligand and Fas receptor in cortical neurons transfected with a gene encoding Fas ligand (see Materials and Methods). E16 cortical neurons were transfected with a recombinant adenoviral vector with or without Fas ligand. At 48 hr after transfection, cell lysates were subjected to Western blot using an anti-Fas ligand antibody (N-20) or an anti-Fas antibody (M-20). Fas receptor was constitutively expressed and did not change, whereas Fas ligand was upregulated in neurons transfected with vector containing the gene encoding Fas ligand (rAd–FasL) but not by control vector (rAd–LacZ). *B*, Coimmunoprecipitation of Fas–procaspase-8 in cultured cortical neurons transfected with Fas ligand. E16 neurons were transfected with adenoviral vectors as in *A*. At 48 hr after transfection, cell lysates were incubated with anti-caspase-8 antibody (H-134), and the immunoprecipitants were separated by SDS-PAGE and probed with anti-Fas antibody (clone 13). Fas–procaspase-8 coimmunoprecipitation was increased in overexpressing cortical neurons. *IP*, Immunoprecipitation antibody. *C*, Neuronal death induced by Fas ligand *in vitro* is mediated in part by caspases. Cell death was assessed at 72 hr in cortical neurons overexpressing Fas ligand (trypan blue staining). Eighty percent of neurons transfected with Fas ligand died (*vertical stripes*) versus control vector (*diagonal stripes*) ($^*p < 0.05$). Pretreatment with ZVAD-fmk (500 μM; *open bar*) reduced Fas ligand-induced cell death by ~50% at 72 hr after transfection compared with vehicle (0.5% DMSO; *solid bar*) ($^{**}p < 0.05$ vs control).

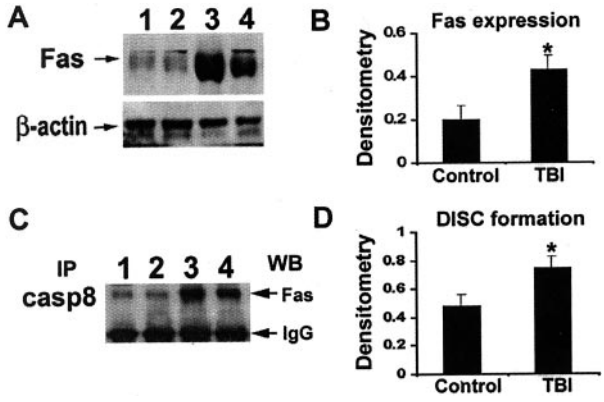

Figure 6. Fas expression and DISC formation is increased in contused human brain. *A*, Western blot analysis of brain tissue homogenates from representative patients with severe TBI (*lanes 3* and *4*) or refractory seizures (*lanes 1* and *2*) or of postmortem tissue from patients dying of non-CNS causes ($n = 1$). Brain homogenates were separated by SDS-PAGE and Western blots were probed with anti-Fas antibody (C-20). Fas receptor expression was increased in patients with TBI. β-actin was also probed to confirm equal protein loading. *B*, Densitometric analysis of data from all patients with TBI ($n = 5$), seizures ($n = 5$), and non-CNS causes of death ($n = 1$). $^*p < 0.05$ versus control. *C*, Fas–procaspase-8 interaction assessed by immunoprecipitation of homogenates from patients with severe TBI (*lanes 3* and *4*) or refractory seizure disorders (*lanes 1* and *2*) as described in *A*. Cortical homogenates were incubated with anti-procaspase-8, and the immunoprecipitants were subjected to SDS-PAGE. Immunoblots were probed with antibodies against human Fas receptor (clone 33). Coimmunoprecipitation of Fas–procaspase-8 is apparent in patients with TBI. Bands corresponding to light-chain IgG were of equal intensity in all *lanes*, suggesting equal antibody loading. *D*, Densitometric analysis of Fas–procaspase-8 immunoprecipitants was performed as in *B*. Fas–procaspase-8 coimmunoprecipitation is increased in brains taken from patients with TBI ($^*p < 0.05$ vs control). *IP*, Immunoprecipitation antibody; *WB*, immunoblotting antibody.

2000a). The present study is the first to demonstrate linkage between ligation and activation of Fas, Fas-associated DISC assembly, and activation of initiator and effector caspases after acute TBI. We found that Fas receptors were expressed by neurons after CCI in mice, and also found evidence for Fas–Fas ligand interaction and formation of Fas–FADD–procaspase-8, Fas–FADD–procaspase-10, and Fas–RIP–RAIDD–procaspase-2 complexes preceding the onset of significant cell death. Early DISC assembly was followed by processing of caspase-8 and caspase-3 and both Fas and Fas ligand colocalized to TUNEL-positive neurons after CCI. DISC formation was found in cultured neurons when Fas ligand was overexpressed. A majority of the cells were killed by 72 hr, and cell death was significantly inhibited by the application of a pan-caspase inhibitor. The coimmunoprecipitate of Fas–procaspase-8 was also found in human brain after severe traumatic brain injury, suggesting common cell-death mechanisms in more than a single mammalian species. Together, the data suggest that Fas may function as a death receptor in mammalian brain, and that death-receptor activation may provide an important initiating mechanism of neuronal death after TBI.

Neurons appear to be the most likely site for Fas expression and DISC assembly. Infiltrating leukocytes can be a source of Fas and Fas ligand and DISC proteins, but we did not detect neutrophils at early times of DISC assembly in contused brain (3–6 hr), and lymphocyte accumulation in contused brain occurs after 24 hr (Holmin et al., 1995). Furthermore, DISC assembly was always greater in contused mouse brain than in brain injected with an equivalent amount of blood. Finally, the magnitude of Fas–procaspase-8 immunoprecipitant did not relate in any simple way to blood content in the human TBI samples (data not shown). Nevertheless, our data do not rule out other sites of Fas expression and DISC assembly. Both Fas and Fas ligand expressed on microglia and astrocytes may play important roles in cell killing in the brain (Saas et al., 1999; Aquaro et al., 2000; Lee et al., 2000). Thus, Fas ligand induced on glia could cause Fas-mediated autocrine cell death of glia or paracrine neuronal cell death. Our data showing colocalization of Fas and Fas ligand in neurons are consistent with an autocrine mechanism of neuronal cell death. Additional study is required to determine the role of glia in Fas-mediated cell death after TBI.

The most notable finding here is that DISC assembles early and is robust in contused brain after both experimental and human TBI. DISC formation does not appear to be a stereotyped response to acute CNS injury but may reflect unique features of TBI. For example, the robustness of DISC assembly after TBI appeared far greater than that found in ischemic spinal cord (Matsushita et al., 2000) or brain after ischemia/reperfusion, even when assessed during the evolution of ischemic injury (M. A. Moskowitz, unpublished observations). Such differences may reflect unique aspects of

pathophysiology such as specific gene expression and protein synthesis or recruitment and activation of distinct cell types between TBI and ischemia. Moreover, we and others have reported rapid upregulation of Fas receptor within minutes after CCI (Beer et al., 2000a) (data not shown), but only after several hours of reperfusion after cerebral or spinal cord ischemia in mice (Martin-Villalba et al., 1999; Matsushita et al., 2000; Rosenbaum et al., 2000). Mechanisms governing the unusually rapid increase in Fas receptor expression after TBI remain to be elucidated.

DISC assembly was associated with robust cleavage of caspase-8 to its 18 kDa (active) fragment as detected in brain homogenates by 3 hr and in TUNEL-positive neurons by 48 hr after CCI (Fig. 4). Furthermore, we detected cytochrome *c* release at later times (12–48 hr) after CCI (data not shown). We and others detected the 18 kDa caspase-8 fragment in spinal motoneurons at 1.5 hr after transient ischemia (Matsushita et al., 2000), in rat cortical neurons 6 hr after permanent ischemia (Velier et al., 1999), and in rat brain homogenates within several hours of hypoxic/ischemic brain injury (Northington et al., 2001) and fluid percussion TBI (Keane et al., 2001). In agreement with our results, caspase-3 cleavage, as assessed by immunohistochemistry, appeared early but was maximal at 48 hr after CCI in rats (Beer et al., 2001). Our data are consistent with a cell-death mechanism early on in which caspase-8 induces cell death by directly cleaving and activating caspase-3 (type I) rather than via release of cytochrome *c* from mitochondria (type II) (Scaffidi et al., 1998, 1999). Type I cell death may develop in ischemic spinal motoneurons (Matsushita et al., 2000). Type II cell death requires mitochondrial amplification of caspase-8 cleavage by a positive feedback loop involving caspase-8-mediated processing of bid, release of mitochondrial cytochrome *c*, and activation of caspase-3, which then may cleave and activate caspase-8. Indirect evidence for type II cell death was reported after TBI (Keane et al., 2001) and derives from studies showing that overexpression of Bcl-2, an anti-apoptotic protein that inhibits cytochrome *c* release, reduces contusion volume after CCI in transgenic mice (Raghupathi et al., 1998; Nakamura et al., 1999).

We detected increased Fas protein and marked upregulation of Fas–procaspase-8 interaction in contused brain removed from patients with severe TBI, particularly at later times after injury. Although the sample size limited more detailed analysis, Fas upregulation and DISC formation were significantly greater in contused brain than in brain removed from humans with refractory seizures or tissue removed postmortem from a patient who died of non-CNS causes. Although both diseases are associated with death receptor-mediated mechanisms, the differences between results from contused brain homogenates were striking. The data suggest that DISC formation is relevant to human TBI and in part, validate studies in the mouse. The observation that Fas forms a DISC with procaspase-2, procaspase-8, and procaspase-10 suggests that Fas may activate other initiator caspases after TBI. Fibroblasts from caspase-8-deficient mice are completely resistant to death induced by Fas, tumor necrosis factor receptor 1, and death receptor 3 signaling, suggesting a central role for caspase-8 in non-neuronal cell types. Whether caspase 8 mediates cell death after CNS injury cannot be studied in mutant mice directly, because the caspase-8 null mutation is lethal at day 12.5 of embryogenesis (Varfolomeev et al., 1998). Humans with loss-of-function mutations in caspase-10 have autoimmune lymphoproliferative syndromes but no known CNS phenotype in noninjured brain (Wang and Lenardo, 2000). Caspase-2 mediates the cell death induced by trophic deprivation and β-amyloid in cultured neurons (Stefanis et al., 1997; Troy et al., 1997, 2000; Haviv et al., 1998). However, neurons lacking caspase-2 remain sensitive to cerebral ischemia and facial nerve axotomy *in vivo* and trophic deprivation *in vitro* (Bergeron et al., 1998), likely because of a compensatory overexpression of caspase-9 (Troy et al., 2001). Whether caspase-2 plays a role in post-traumatic brain cell death requires additional study.

Our data confirm that Fas and Fas ligand are constitutively expressed in the adult mouse brain (Park et al., 1998), and that Fas and Fas ligand colocalize to neurons after CCI (Beer et al., 2000a). To pursue mechanisms of Fas-mediated neuronal cell death in more detail, we overexpressed Fas ligand using an adenoviral vector to determine whether Fas receptor is engageable by neuronally expressed ligand and capable of promoting cell death via DISC formation. Compared with treatment with empty vector, overexpression of Fas ligand induced Fas–FADD–procaspase-8 DISC assembly and a fourfold increase in cell death mediated in part by caspases. In contrast, addition of soluble Fas ligand to cultured neurons failed to cause cell death (preliminary data not shown), suggesting that activation of neuronal Fas by extrinsic Fas ligand may be less efficient than when endogenously expressed.

We also identified death-receptor mechanisms in TBI that are common to both apoptosis and necrosis. RIP is a protein kinase that is activated by Fas signaling with both proapoptotic and anti-apoptotic actions. Transient RIP overexpression promotes apoptosis (Stanger et al., 1995), but RIP may also promote necrosis in non-neuronal cell types, presumably by phosphorylating key regulatory proteins (Holler et al., 2000). Caspases are important in Fas-mediated cell killing (Cheema et al., 1999; Raoul et al., 1999); however, Fas signaling can also kill cells by caspase-independent mechanisms that resemble necrosis (Kawahara et al., 1998; Vercammen et al., 1998; Matsumura et al., 2000). Necrosis induced by Fas ligand in T lymphocytes requires functional FADD and RIP (Holler et al., 2000). Because necrosis and apoptosis cause cell death after TBI, death receptor-mediated events that converge at the level of the DISC might activate both apoptotic and necrotic cell-death mechanisms in injured neurons. If so, then inhibition of DISC formation or function could potentially impact multiple mechanisms governing post-traumatic and perhaps other forms of neuronal death.

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