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Immunohistochemical characterization of Fas (CD95) and Fas Ligand (FasL/CD95L) expression in the injured brain: Relationship with neuronal cell death and inflammatory mediators

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Summary. Traumatic brain injury causes progressive tissue atrophy and consequent neurological dysfunction, resulting from neuronal cell death in both animal models and patients. Fas (CD95) and Fas ligand (FasL/CD95L) are important mediators of apoptosis. However, little is known about the relationship between Fas and FasL and neuronal cell death in mice lacking the genes for inflammatory cytokines. In the present study, double tumor necrosis factor/lymphotoxin-α knockout (–/–) and interleukin-6–/– mice were subjected to closed head injury (CHI) and sacrificed at 24 hours or 7 days post-injury. Consecutive brain sections were evaluated for Fas and FasL expression, in situ DNA fragmentation (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; TUNEL), morphologic characteristics of apoptotic cell death and leukocyte infiltration. A peak incidence of TUNEL positive cells was found in the injured cortex at 24 hours which remained slightly elevated at 7 days and coincided with maximum Fas expression. FasL was only moderately increased at 24 hours and showed maximum expression at 7 days. A few TUNEL positive cells were also found in the ipsilateral hippocampus at 24 hours. Apoptotic, TUNEL positive cells mostly co-localized with neurons and Fas and FasL immunoreactivity. The amount of accumulated polymorphonuclear leukocytes and CD11b positive cells was maximal in the injured hemispheres at 24 hours. We show strong evidence that Fas and FasL might be involved in neuronal apoptosis after CHI. Furthermore, Fas and FasL upregulation seems to be independent of neuroinflammation since no differences were found between cytokine–/– and wild-type mice.

Key words: Apoptosis, Fas, Cytokines, Closed head injury, TUNEL, Neurodegeneration, Traumatic brain injury

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Introduction

Over the past few years, the mechanisms of neuronal cell death after traumatic brain injury (TBI) have received increased attention, particularly due to the high vulnerability of neurons to chronic degeneration. Neuronal cell death resulting from trauma is a complex phenomenon possibly generating from the simultaneous activation of different molecular cascades in response to trauma which remain effective for a long time period and cause progressive tissue atrophy and neurological impairment (Smith et al., 1997; Conti et al., 1998; Fox et al., 1998). Evidence for apoptotic and necrotic cell death of neurons and glial cells has been reported in the brain of rodents subjected to fluid percussion injury and controlled cortical impact injury (Rink et al., 1995; Conti et al., 1998; Kaya et al., 1999; Clark et al., 2000) as well as in head trauma patients (reviewed by: Raghupathi et

al., 2000).

Two pathways of apoptosis, referred to as intrinsic and extrinsic, have been recognized as the two major pathways of caspase-induced cell death and evidence exists for the involvement of both pathways in neuronal cell death following TBI. The extrinsic pathway is initiated by the binding of Fas (CD95) with either its natural ligand FasL (CD95L) or its agonistic anti-Fas antibody (Suda and Nagata, 1994; Becher et al., 1998; Martin-Villalba et al., 1999; Felderhoff-Mueser et al., 2000). Fas is a type I transmembrane receptor glycoprotein belonging to the nerve growth factor (NGF)/tumor necrosis factor (TNF)/lymphotoxin (LT)-α receptor family (Nagata and Golstein, 1995; Cheema et al., 1999; Nagata, 1999; Rosenbaum et al., 2000). FasL is a type II membrane protein of the TNF family. Constitutive cerebral expression of Fas and FasL has been previously shown in humans and rodents (Park et al., 1998; Bechmann et al., 1999) and its upregulation was demonstrated in various neuropathologies including TBI (Dowling et al., 1996; D'Souza et al., 1996; Bonetti and Raine, 1997; Saas et al., 1997; Sabelko et al., 1997; Waldner et al., 1997; Zipp et al., 1997).

Following controlled cortical impact injury, the increased expression of Fas and FasL was identified on cortical neurons and astrocytes (Beer et al., 2000, 2001; Qiu et al., 2002). Interestingly, the activation of Fas and caspase-3 and -8 was found to correspond temporally in response to focal brain damage, suggesting a downstream involvement of the extrinsic pathway of apoptosis (Keane et al., 2001; Qiu et al., 2002). Our group and others have previously detected elevated concentrations of soluble Fas (sFas) and FasL in human cerebrospinal fluid (CSF) following severe TBI (Ertel et al., 1997; Lenzlinger et al., 2002). Particularly in a study of our group, the prolonged release of sFas in the CSF of patients with severe head trauma strongly correlated with the presence of neuron specific enolase, a marker of neuronal damage, corroborating the hypothesis that Fas might play a potential role in the mechanisms of neuronal apoptosis (Lenzlinger et al., 2002).

It is a matter of controversy whether cerebral inflammation initiated after TBI contributes to neurodegeneration or rather to the processes of tissue repair (reviewed by: Morganti-Kossmann et al., 2001; Nagata and Golstein, 2001). Over the past decade, the profound cerebral inflammatory response following clinical or experimental TBI has been extensively described (Schoettle et al., 1990; Fan et al., 1996; Lassmann, 1997; Stahel et al., 1998; Penkowa et al., 1999; Sherwood and Prough, 2000; Morganti-Kossmann et al., 2000, 2002). In particular, the role of the cytokines TNF (Taupin et al., 1993a; Morganti-Kossmann et al., 1997; Shohami et al., 1999; Sullivan et al., 1999; Venters et al., 2000), interleukin (IL)-6 (Woodroofe et al., 1991; Taupin et al., 1993a,b) and other immune mediators (Rothwell and Hopkins, 1995; Sei et al., 1995; Holmin et al., 1997; Feuerstein et al., 1998; Ghirnikar et al., 1998; Morganti-Kossmann et al., 2000) has been the focus of several studies.

Although apoptosis is considered to be a process of cell death independent from immunoactivation, apoptotic cell death of neurons has been observed at points in time which corresponded to the elevation of intracerebral cytokine production after experimental head injury in mice (Grosjean et al., 2001; Morganti-Kossmann et al., 2002). Moreover, the structural homology of both the TNF and Fas receptors, together with the existence of a common intracellular apoptotic cascade, suggests a potential link between cytokines and cell death. In support of this is the evidence that cytokines such as TNF and IL-6 have the ability to modulate Fas and FasL expression in cultured astrocytes (Choi et al., 1999).

In a model of closed head injury (CHI), neurotoxic properties have been attributed to TNF since its elevation in the brain was associated with tissue damage, edema formation, neurological impairment and blood-brain barrier dysfunction. These adverse effects were attenuated by the therapeutical application of agents neutralizing the action of TNF (reviewed by: Shohami et al., 1999). However, more recently, the use of TNF knockout(–/–), TNF/LT-α double–/– or TNF receptor–/– mice in models of focal brain injury have raised conflicting results as novel neuroprotective functions have been ascribed to TNF (Scherbel et al., 1999; Sullivan et al., 1999; Stahel et al., 2000). Opposed to TNF, IL-6 has been shown to possess a protective role for the cells of the nervous system either due to intrinsic neurotrophic properties or through the induction of NGF and the inhibitory action of potentially neurotoxic cytokines such as TNF (Aderka et al., 1989; Hama et al., 1989, 1991; Kossmann et al., 1996).

In the present study, we investigated whether the lack of TNF, LT-α and IL-6 gene expression influences neuronal cell death, leukocyte infiltration and the time profile of Fas and FasL protein expression in the brain of cytokine–/– mice and corresponding wild-type littermates subjected to experimental CHI.

Materials and methods

Animals

Mice double-deficient in genes for TNF and LT-α (TNF/LT-α–/–) or deficient in the IL-6 gene (IL-6–/–) and the corresponding wild-type littermates were provided by H.-P. Eugster (Eugster et al., 1996) and M. Kopf (Kopf et al., 1994). The TNF/LT-α–/– mice were from a mixed C57BL/6x129Sv/Ev (B6x129) genetic background (Eugster et al., 1996), whereas the IL-6–/– animals were backcrossed for ten generations to a C57BL/6 (B6) background (M. Kopf, personal communication). B6x129 (n=7) mice were used as control animals for the TNF/LT-α–/– (n=7) mice and B6 (n=8) mice as control animals for the IL-6–/– (n=7) mice. Sham-operated mice (n=4, one for every genetic group) and normal wild-type mice (n=4) were used as

additional control animals. All mice used in this study (n=37) were males aged 8 to 16 weeks, with an average weight of 28 to 32 g. They were bred in a specific pathogen-free environment, kept under standard conditions of temperature and light in cages of four to six mice and fed with food and water *ad libitum*. The animal experiments were performed in accordance with the guidelines of the Institutional Animal Care Committee of the Hebrew University of Jerusalem, Israel.

Experimental brain injury

Experimental CHI was performed in mice (total n=29) as previously described by Chen et al. (1996). In brief, the mice were anesthetized with ether and their skull was exposed by a longitudinal incision of the skin. A focal trauma was delivered to the closed skull of the left hemisphere 2 mm lateral to the midline in the midcoronal plane using an electric weight drop device with a metal rod of 333 g falling from a height of 2 cm. A silicone tip of 3 mm diameter was fixed at the end of the impacting rod in order to avoid penetrating skull fractures. After injury, mice received temporary oxygen support with 95% O_2 until awake and were then brought back to their cages with food and water *ad libitum*. Sham-operated mice were treated the same way as the injured animals but were not subjected to CHI. For histology, animals were sacrificed by decapitation under ether anesthesia at 24 hours and 7 days following experimental CHI. The brains were immediately removed, both hemispheres separated, snap-frozen in liquid nitrogen, embedded in OCT compound (Tissue-Tek, Miles Inc., Elkhart, IN) and stored at -70°C until further analysis. Sham-operated mice were sacrificed at 24 hours following sham operation and their brains were processed as mentioned above. Three independent series of six serial coronal sections were performed. Every first section was double-stained with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and bisbenzimide Hoechst 33342 (HO33342) fluorochrome in order to study cell death and the morphology of the affected cells and brain tissue. Every second to sixth section underwent immunohistochemistry (IHC) using the respective antibodies and methyl green counterstaining as described below. The tissue sections were analyzed simultaneously in order to allow a direct comparison of the stainings between the different animal groups and different points in time.

The time points for subject sacrifice and brain examination were determined in our laboratory through the results of a preliminary study in which the brains of subject mice were assessed for TUNEL positive cells at 2, 4, 12, 24 hours and 7 days following experimental CHI. The maximum level of TUNEL positive cells was found at 24 hours and the minimum level at 7 days (data not shown). On the basis of this evidence we selected the two points in time, 24 hours and 7 days post-trauma, for further immunohistochemical analysis as described below. Furthermore, it has been previously shown that the extent of PMN infiltration (Biagas et al., 1992; Shapira et al., 1993; Clark et al., 1994; Stahel et al., 2000) and of cell death (Conti et al., 1998; LaPlaca et al., 1999; Beer et al., 2000, 2001; Raghupathi et al., 2002) in the mouse and rat brain were both highest at 24 hours post-injury. It has also been previously described that Fas and FasL expression in the injured rat brain was maximal at 24 hours post-trauma (Beer et al., 2000, 2001).

TUNEL and Hoechst 33342 staining

To assess intracranial cell death, DNA fragmentation was visualized by using the in situ cell death detection kit fluorescein (Roche Applied Science, Rotkreuz, Switzerland), which is based on the TUNEL histochemistry technique developed by Gavrieli et al. (1992). The protocol of the kit was used with minor modifications as described below. 10 µm-thick serial coronal cryosections of the left (injured, ipsilateral) and right (contralateral) hemisphere of cytokine–/– mice and the corresponding wild-type littermates were analyzed after experimental CHI (t=24 hours, total n=13; t=7 days, total n=16) and in the hemispheres of sham-operated (t=24 hours; n=4) and normal wild-type (n=4) mice. The sections were fixed in 10% neutral buffered formaldehyde solution in phosphate-buffered saline (PBS) for 10 minutes at room temperature (RT), differentiated in 2:1 ethanol:acetic acid solution for 5 minutes at -20°C and permeabilized in 3% Triton X-100 in PBS for 60 minutes at RT. The sections were then incubated in equilibration buffer (30 mM Tris pH 7.2, 140 mM cacodylate sodium salt, 1 mM CoCl2, in dH_2O) of the TdT enzyme for 20 minutes at RT. Labeling was performed in humidified chambers by the application of the TUNEL reaction mixture for 90 minutes at 37°C. To visualize the total amount of cells, HO33342 (Calbiochem, San Diego, CA), an UV excited blue bisbenzimidiazole dye, which selectively intercalates into A-T rich regions of DNA and therefore stains all nuclei (Pollack and Ciancio, 1990), was added simultaneously to the TUNEL mixture. HO33342 dye is often used to distinguish condensed pycnotic nuclei in apoptotic cells. Finally, the sections were mounted with De Pe X (BDH Laboratory Supplies, Poole, Great Britain). Both TUNEL and HO33342 positive cells were then visualized by fluorescence microscopy using an Olympus BH-2 microscope (Olympus Optical Co., Hamburg, Germany). For negative controls, the terminal TdT enzyme was omitted. TUNEL positive cells were assigned to be apoptotic or non-apoptotic based on specific verification of two or more of the classic morphological hallmarks of apoptosis: chromatin condensation and margination, nuclear shrinkage and fragmentation, formation of apoptotic bodies and cytoplasm condensation. TUNEL positive cells showing nuclei with diffuse staining and without apoptotic

morphology were considered to be necrotic.

Immunohistochemistry

Immunohistochemistry was carried out on sections of ipsi- and contralateral hemispheres of cytokine deficient–/– and wild-type mice after CHI (t=24 hours, total n=13; t=7 days, total n=16), after sham operation (t=24 hours, n=4) and on sections of normal wild-type mouse brains (n=4). In brief, the sections were fixed in absolute acetone (Riedel-de Hafin, Seelze, Germany) for 5 minutes at RT, blocked in a buffer containing 4% cow's milk and 2% horse serum in PBS for 1 hour at RT and incubated with the primary antibodies diluted in 2% cow's milk in PBS as described below. Neurons were identified by using a mouse monoclonal anti-neuronal nuclei (NeuN, 1:100; Chemicon, Temecula, CA) antibody. For immunolabeling for Fas or FasL protein expression, the respective sections were incubated overnight at 4°C with rabbit polyclonal anti-mouse Fas antibody (M-20, 1:1000; Santa Cruz Biotechnology, CA) or with goat polyclonal anti-mouse FasL antibody (N-20, 1:750; Santa Cruz Biotechnology). Infiltrating polymorphonuclear leukocytes (PMNs) were stained using a rat anti-mouse polymorphonuclear leukocyte antibody (1:100; BioSource, Camarillo, CA). Macrophages and activated microglia were visualized by using a rat monoclonal anti-mouse complement receptor type 3 antibody (CD11b, 1:100; Pharmingen, San Diego, CA). Detection of the primary antibodies was performed by using peroxidase-coupled secondary antibodies and the Vectastain ABC kit (Vector Labs, Burlingame, CA) with 3,3'-Diaminobenzidine tetrahydrochloride as chromogen. The secondary biotinylated antibodies were from Vector or BioSource and were applied for 1 hour at RT at the dilutions 1:200 (anti-mouse IgG), 1:400 (anti-rabbit IgG and anti-goat IgG) and 1:500 (anti-rat IgG) in 2% cow's milk in PBS. In negative controls, the primary antibody was omitted and the sections were incubated with blocking buffer. Methyl green was used as counterstain on all sections.

Analysis of Fas and FasL expression and accumulation of leukocytes

For assessment of intracranial Fas and FasL expression, leukocyte accumulation and degeneration of neurons after trauma, double staining with IHC and methyl green was performed on sections of mouse brains at 24 hours and 7 days following experimental CHI as described above. Fas and FasL positive cells in both the cortex and hippocampus were counted for each animal on the side of injury (left) and on the contralateral side (right) at 20x magnification (final magnification 250x) using a stereological grid in the ocular lens of the microscope. For each section, the counts were performed in four randomly selected fields of 0.4 mm^2 within the penumbra of the injury in the deeper cortical layers below the directly contused area and within the CA2/3 regions of hippocampus. The corresponding total cell number was determined by counting all methyl green-counterstained cells in the identical fields. For correction of differences in the cell size of normal and apoptotic nuclei, apoptotic nuclear fragments that were enfolded by methyl green-stained nuclear contours were regarded as one nucleus. The level of Fas and FasL expression in defined areas of the cortex and hippocampus was assigned to one of four categories:

– =	no staining or occasional weak staining
+ =	few Fas or FasL positive cells (<10%)
++ =	10% to 50% Fas or FasL positive cells
+++	= 50% to 90% Fas or FasL positive cells
++++	= 90% to 100% Fas or FasL positive cells.

In order to determine the extent of leukocyte and macrophage accumulation, microglia activation and neuron degeneration in the injured hemispheres, PMNs and CD11b and NeuN positive cells were quantified on consecutive sections as described above. For each section the counts were carried out in four randomly selected fields of 0.4 mm^2 in the deeper cortical layers below the direct contusion site and in the corresponding sites of the contralateral hemispheres. Cells were also counted in equivalent areas of the brains of control mice. The corresponding total cell number was determined by counting all methyl green-counterstained cells. The percentages of PMNs and CD11b positive cells were calculated and expressed as mean value ± standard deviation. In all experiments, the corresponding tissue sections were analyzed simultaneously in order to allow a direct comparison of the stainings in the different animal groups at the different points in time.

Statistical analysis

Statistical significance of differences between the examined groups was analyzed using the unpaired two-sided Student's t-test. A probability of $P < 0.05$ was considered statistically significant.

Results

Analysis of regional and temporal distribution of DNA fragmentation

Cell death was assessed by TUNEL staining (Fig. 1A,C,E,G) and the total number of cells was visualized by simultaneous staining with HO33342 dye (Fig. 1B,D,F,H). Brain sections of both normal wild-type mice and sham-operated control mice (24 hours following skin incision) showed no significant amount of TUNEL positive cells (Fig. 1A). In addition, the staining with HO33342 dye (Fig. 1B) showed normal nuclear morphology in the sections of the control mouse brains. In the traumatized mice, 24 hours after experimental CHI, the injured parieto-temporal cortex was characterized by the presence of a striking amount of

TUNEL positive cells which were present also in deeper located, not directly contused cortical layers, the so-called penumbra zone of the lesion (Fig. 1C, arrowheads), being of similar extent in both –/– and wild-type mice. Staining with HO33342 dye also revealed the presence of condensed nuclei within the deeper cortical layers (Fig. 1D, arrowheads), suggesting nuclear condensation, one of the morphological hallmarks of apoptosis. Interestingly, we also found a few TUNEL positive cells in the ipsilateral hippocampus within the CA2/3 regions at 24 hours post-trauma (Fig. 1E) whereas TUNEL positive cells were virtually undetectable in the hippocampus at 7 days post-trauma (see Fig. 4I). The amount of TUNEL positive cells in the

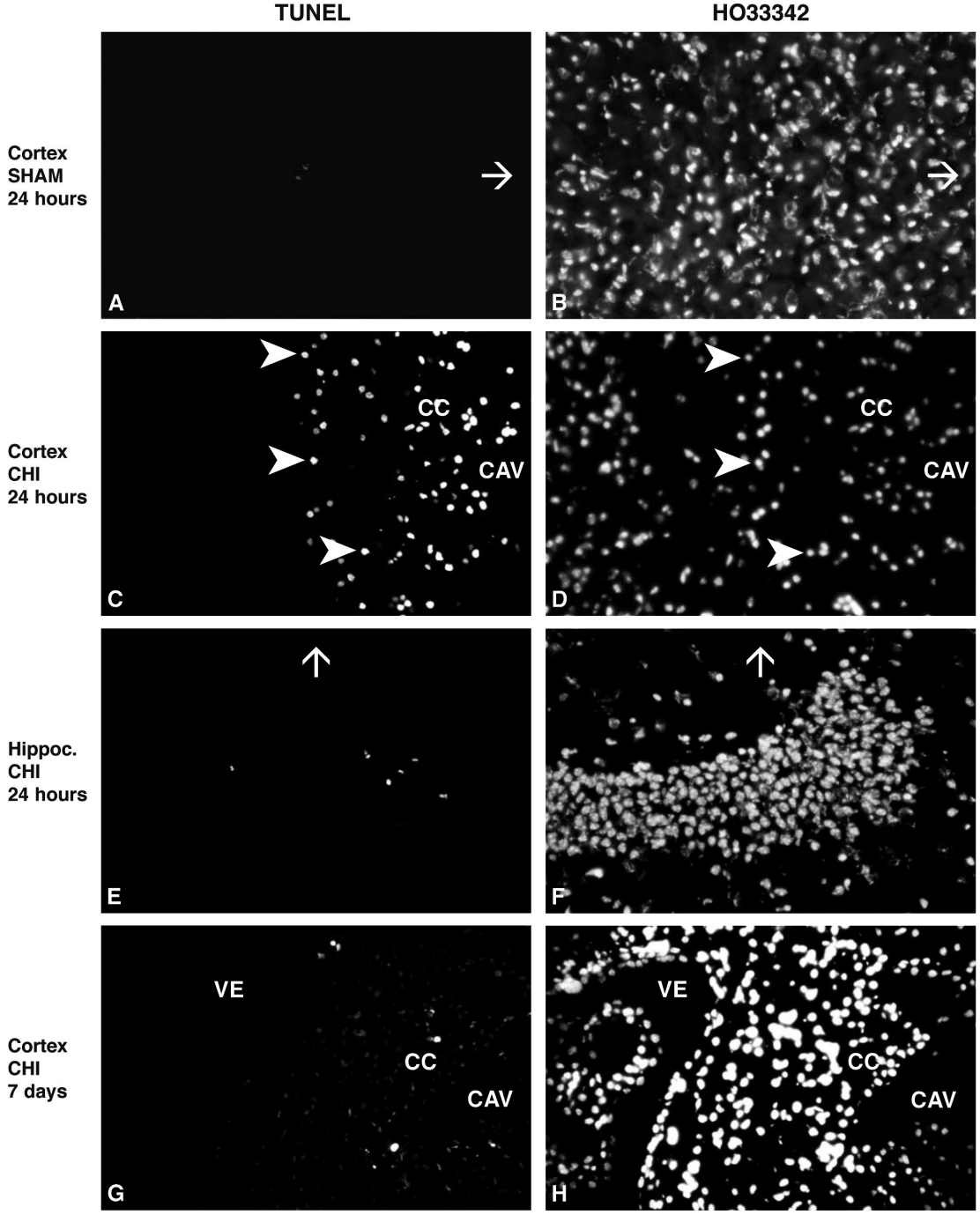

Fig. 1. Cell death in the mouse brain following closed head injury. Brain sections of 10 mm were analyzed at 24 hours and 7 days after experimental CHI using TUNEL/HO33342 fluorescence double staining. Representative photomicrographs are shown for B6–/– mice and demonstrate pairs of identical views using different UV filters. The left column shows TUNEL staining: cortex at 24 hours after sham operation **(A)** and at 24 hours after CHI **(C)**, hippocampus at 24 hours after CHI **(E)** and cortex at 7 days after CHI **(G)**. Note the maximum extent of TUNEL positive cells in and adjacent to the directly contused cortex (CC) at 24 hours **(C)** and the decreased extent of cell death at 7 days following CHI **(G)**. The right column displays the total amount of cells by HO33342 staining (**B, D, F and H)** in the identical fields as shown for TUNEL staining. The arrowheads point to deeper cortical layers not directly contused by the impact. The arrows point towards the brain surface. CHI: closed head injury, –/–: knockout, SHAM: sham-operated, Hippoc.: hippocampus, CAV: contusion cavity, VE: ventricle. A-D,G,H, x 250; E,F, x 500

injured cortex generally decreased at 7 days after trauma (Fig. 1G). However, at this late point in time we found pronounced morphological alterations within the ipsilateral hemispheres such as enlarged ventricles and an increased size of the contusion cavities (Fig. 1H). The contusion cavities expanded into deeper cortical layers at 7 days post-injury (Fig. 1H) as a result of ongoing tissue degradation. Virtually no TUNEL positive cells were found in other regions of the ipsilateral hemispheres or in the contralateral hemispheres at both 24 hours and 7 days after CHI (data not shown). In the negative controls for the TUNEL experiments, no signal was found (data not shown).

Morphological characterization of apoptosis in the injured hemispheres

In order to determine whether cell death after CHI resulted from necrosis or apoptosis, brain sections from the injured hemispheres were double-stained with TUNEL and HO33342 dye. Subsequently, the tissues were analyzed in identical fields for both stainings, based on selection criteria of nuclear morphology for apoptosis, as mentioned above. Apoptotic cells were found in all ipsilateral cortical layers near the site of trauma in all mouse groups. Figure 2 shows representative photomicrographs of the injured cortical layers III and IV of IL-6–/– mice. At 24 hours after CHI, most TUNEL positive cells depicted the characteristic morphological hallmarks of apoptosis such as chromatin margination (Fig. 2A), chromatin condensation (Fig. 2C) and formation of apoptotic bodies. The presence of apoptotic bodies (Fig. 2E), nuclear shrinkage and DNA fragmentation were also observed at 7 days post-injury. A few necrotic cells were also found (Fig. 2E). The corresponding views of the identical cells are shown for staining with HO33342 dye and reveal that apoptotic cells were surrounded by cells with normal nuclear morphology which might either not be affected by the trauma or might just be in a very early state of cell death (Fig. 2B,D,F).

Fas expression after closed head injury

Immunohistochemical analysis of both normal wild-type mice and sham-operated mice of all strains revealed that Fas was constitutively expressed at moderate levels in the cortex (Fig. 3A, Table 1), hippocampus (Fig. 3G, Table 1) and thalamus (Table 1). At 24 hours after CHI, Fas expression was clearly elevated in the injured cortex (Fig. 3C, Table 1), hippocampus (Fig. 3I, Table 1) and thalamus (Table 1) when compared to sham-operated or non-traumatized control mice. Maximum Fas expression was detected in the ipsilateral cortex near the administered CHI in both TNF/LT-α–/– (Table 1) and IL-6–/– (Fig. 3C, Table 1) mice at 24 hours after CHI. In all mouse strains, we found an evident increase in the expression of Fas in the ipsilateral hippocampal CA2/3 regions (Fig. 3I) at 24 hours post-trauma when compared to sham-operated or non-traumatized control mice. Fas expression was elevated in the ipsilateral thalamus of both TNF/LT-α–/– and IL-6–/– mice at 24 hours post-trauma (Table 1). At 7 days after CHI, there was a trend of Fas expression to return to basal levels in the cortex of mice of the B6x129 strain (TNF/LT-α–/– and wild-type) (Table 1) whereas it remained at a slightly higher level in the cortex of mice of the B6 strain (IL-6–/– and wild-type; Fig. 3E, Table 1). In the hippocampus, Fas

Table 1. Relative number and distribution patterns of Fas and FasL immunoreactivity in the injured mouse brains.

Mice	n	Condition	Fas expression			FasL expression		
			Cortex	Hippocampus CA2/3	Thalamus	Cortex	Hippocampus CA2/3	Thalamus
Control	8	SHAM and normal	++	++	++	–	–	–
TNF/LT-α–/–	3	CHI, t = 24 hours	++++	+++	+++	++	–	+
B6x129	3	CHI, t = 24 hours	+++	+++	++	++	–	–
TNF/LT-α–/–	4	CHI, t = 7 days	++	++	++	+++	–	+
B6x129	4	CHI, t = 7 days	++	++	+++	+++	+	+
IL-6–/–	3	CHI, t = 24 hours	++++	+++	+++	+	+	++
B6	4	CHI, t = 24 hours	+++	+++	++	+	+	++
IL-6–/–	4	CHI, t = 7 days	+++	++	++	+++	–	++
B6	4	CHI, t = 7 days	+++	++	++	+++	–	++

Post-traumatic Fas and FasL expression in the injured left hemispheres of TNF/LT-α–/– and IL-6–/– mice and the corresponding wild-type littermates at 24 hours and 7 days after experimental CHI. The relative number of both Fas and FasL positive cells was semi-quantitatively assessed in the cortex, hippocampus and thalamus and was expressed as follows: –, no positive cells; +, few positive cells (<10%); ++, 10% to 50% positive cells; +++, 50% to 90% positive cells; ++++, 90% to 100% positive cells. CHI: closed head injury, SHAM: sham-operated, CA2/3: hippocampal CA2/3 regions of Ammon's horn, TNF/LT-α–/–: mice deficient in genes for TNF and LT-α, B6x129: C57BL/6x129Sv/Ev wild-type mice, IL-6–/–: mice deficient in genes for IL-6, B6: C57BL/6 wild-type mice, Control: B6x129, B6, TNF/LT-α–/– and IL-6–/– sham-operated mice and normal wild-type mice.

expression decreased to basal levels in both strains at 7 days following brain injury (Fig. 3K, Table 1). In the ipsilateral thalamus, the variation in Fas immunoreactivity amongst the two points in time was generally minor if compared to the variation in the ipsilateral cortex of corresponding mice. Fas remained upregulated in the ipsilateral thalamus of B6x129 mice (Table 1) while it returned to basal levels in B6, IL-6–/– and TNF/LT-α–/– mice at 7 days following CHI (Table 1) when compared to the respective control animals.

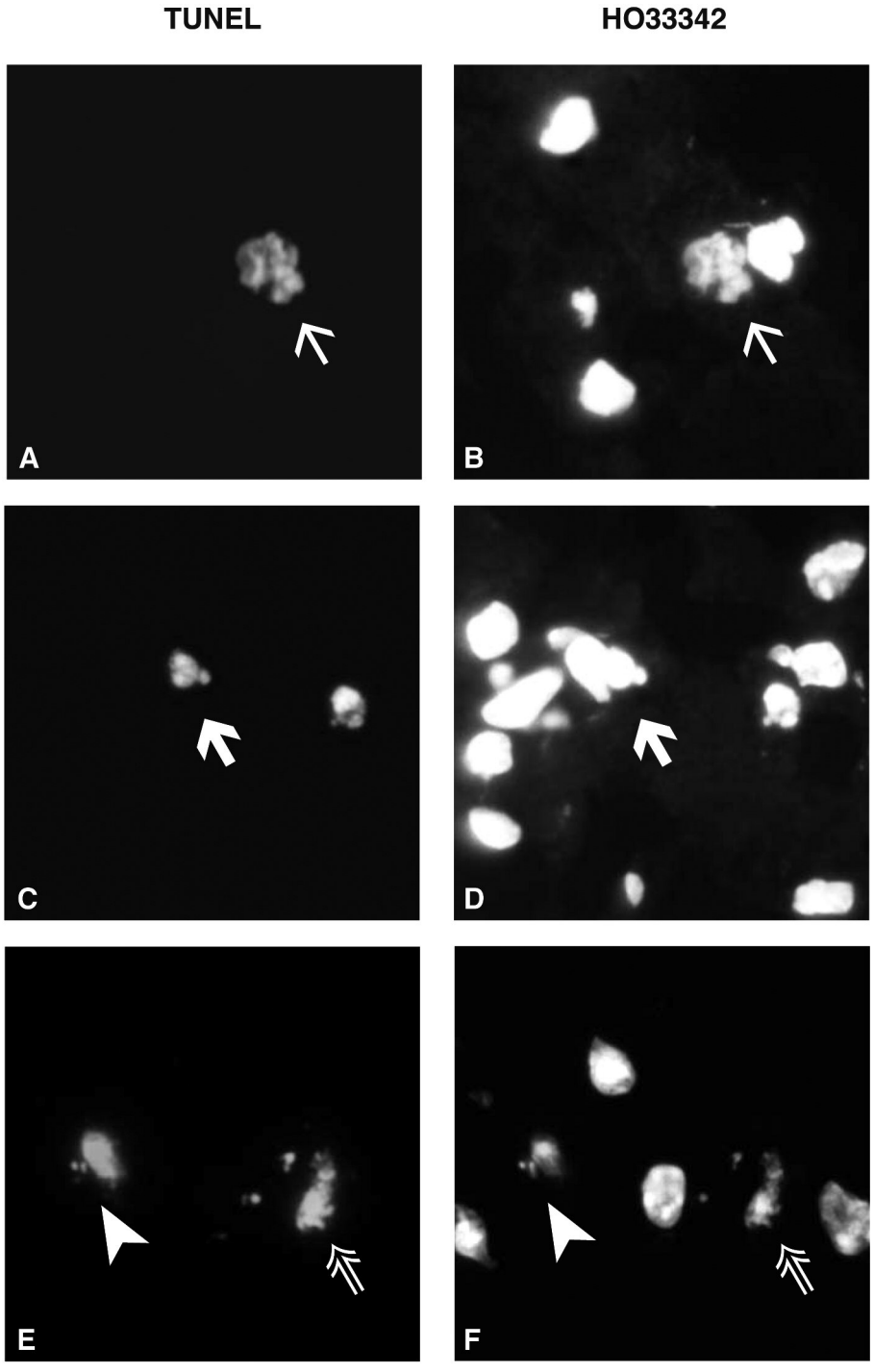

Fig. 2. Apoptosis in the injured cortex. High magnification photomicrographs of the cortical layers III and IV of mice subjected to CHI show TUNEL (**A, C and E**) and HO33342 (**B, D and F**) fluorescence double staining in identical fields representatively for B6–/– mice. Note the presence of chromatin margination (thin arrows) and chromatin condensation (thick arrows) at 24 hours after CHI (**A-D**). The formation of apoptotic bodies is shown here at 7 days after CHI (**E and F**; arrowheads). Necrotic cells depict nuclei with diffuse staining (**E and F**; double arrows) as shown at 7 days post-injury. x 1,250

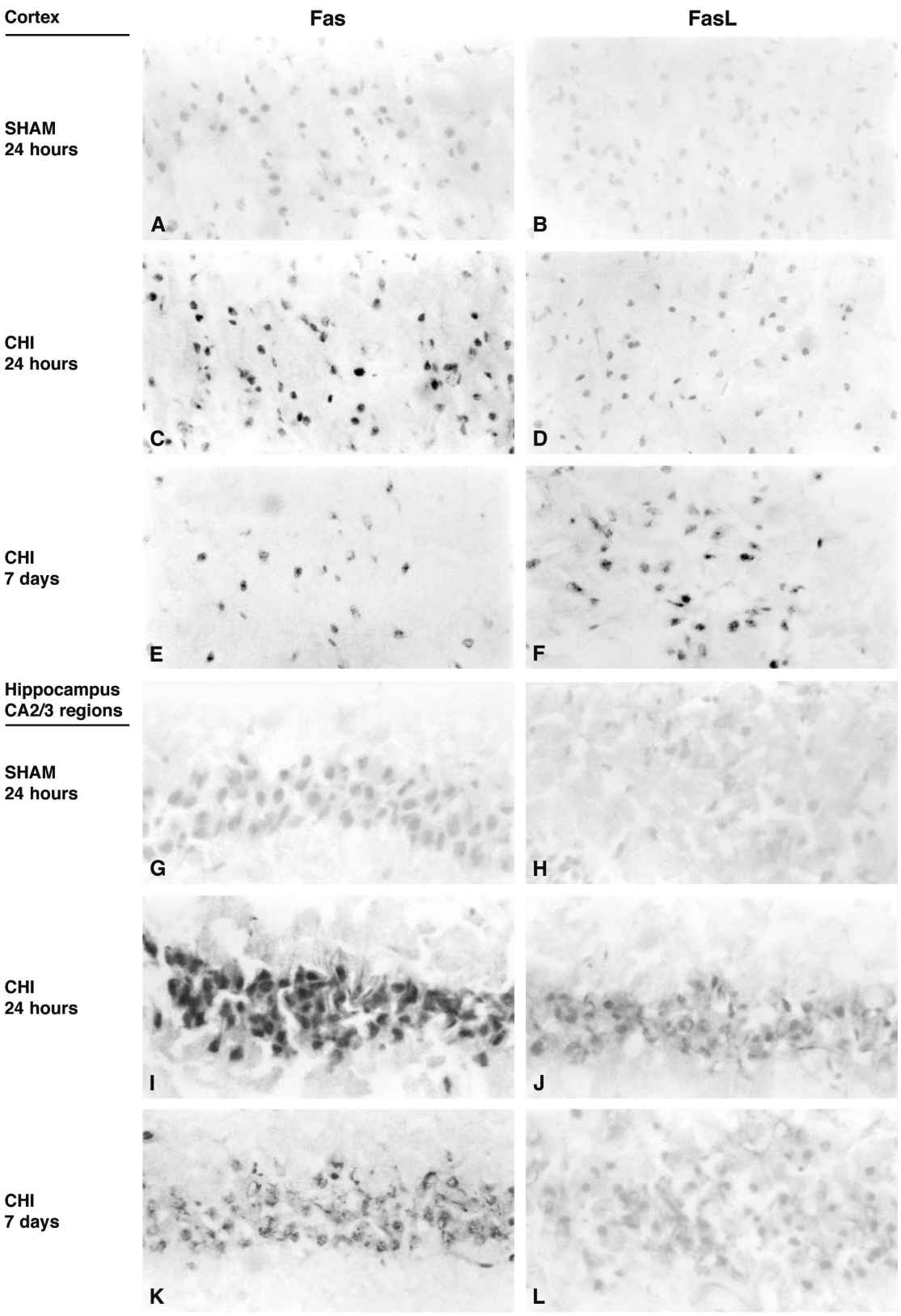

Fig. 3.
Immunolabeling for Fas and FasL and methyl green counterstaining in the injured hemisphere. 10 µm thick consecutive coronal brain sections were analyzed at 24 hours following sham operation or CHI and at 7 days following CHI. Representative photomicrographs for IL-6–/– mice show neurons of the cortex and hippocampus stained for anti-Fas antibody (left column) and anti-FasL antibody (right column). The first part of the figure (**A-F**) shows representative views of the cortex of a sham-operated control mouse (**A and B**) as well as of mice sacrificed at 24 hours (**C and D**) and 7 days (**E and F**) after CHI. The second part of the figure (**G-L**) shows the hippocampal CA2/3 regions of a sham-operated control mouse (**G and H**) as well as of mice at 24 hours (**I and J**) and 7 days (**K and L**) after CHI. Note the constitutive expression of Fas in the cortex (**A**) and hippocampus (**G**) of control mice and the markedly increased amount of stained cells at 24 hours in the cortex **(C)** and hippocampus **(I)** followed by a decrease at 7 days after CHI (**E and K**). In contrast, FasL was virtually undetectable in control brains (**B and H**), was slightly upregulated in the injured brains at 24 hours (**D and J**) and reached maximum upregulation at 7 days (**F and L**) after CHI. CHI: closed head injury, SHAM: sham-operated. x 500

FasL expression after closed head injury

Contrary to the constitutive expression observed for Fas, no FasL immunoreactivity was detected in the cortex (Fig. 3B, Table 1), hippocampus (Fig. 3H, Table 1) or thalamus (Table 1) neither in sham-operated nor in uninjured control mice. At 24 hours following experimental CHI, FasL was moderately increased in the ipsilateral cortex of both the B6x129 mouse strain (TNF/LT-α–/– and wild-type; Table 1) and, to a lesser extent, the B6 mouse strain (IL-6–/– and wild-type; Fig. 3D, Table 1) when compared to control mice. A slightly elevated FasL expression was observed at 24 hours post-injury in the ipsilateral hippocampus of IL-6–/– mice (Fig. 3J, Table 1) and the corresponding B6 littermates but neither in TNF/LT-α–/– nor in B6x129 mice. The ipsilateral thalamus showed moderate FasL expression in mice of the B6 mouse strain and a slight FasL expression in TNF/LT-α–/– mice at 24 hours after trauma (Table 1). The extent of FasL expression in mice from different genetic backgrounds showed only minor differences when compared at 24 hours post-injury. Interestingly, the expression of FasL reached maximum levels which were similar in the ipsilateral cortex of all mice brains subjected to CHI (Fig. 3F, Table 1) at 7 days post-trauma. In the ipsilateral thalamus, moderate FasL immunoreactivity (Table 1) was observed at 7 days post-injury. In the ipsilateral hippocampus almost no positive FasL signal (Fig. 3L, Table 1) was found at 7 days following experimental CHI.

TUNEL positive cells co-localize with neurons and with both Fas and FasL immunoreactivity

The cell types susceptible to degeneration following CHI were identified by TUNEL/HO33342 double staining in conjunction with IHC, using anti-NeuN, anti-PMN and anti-CD11b antibodies on consecutive brain

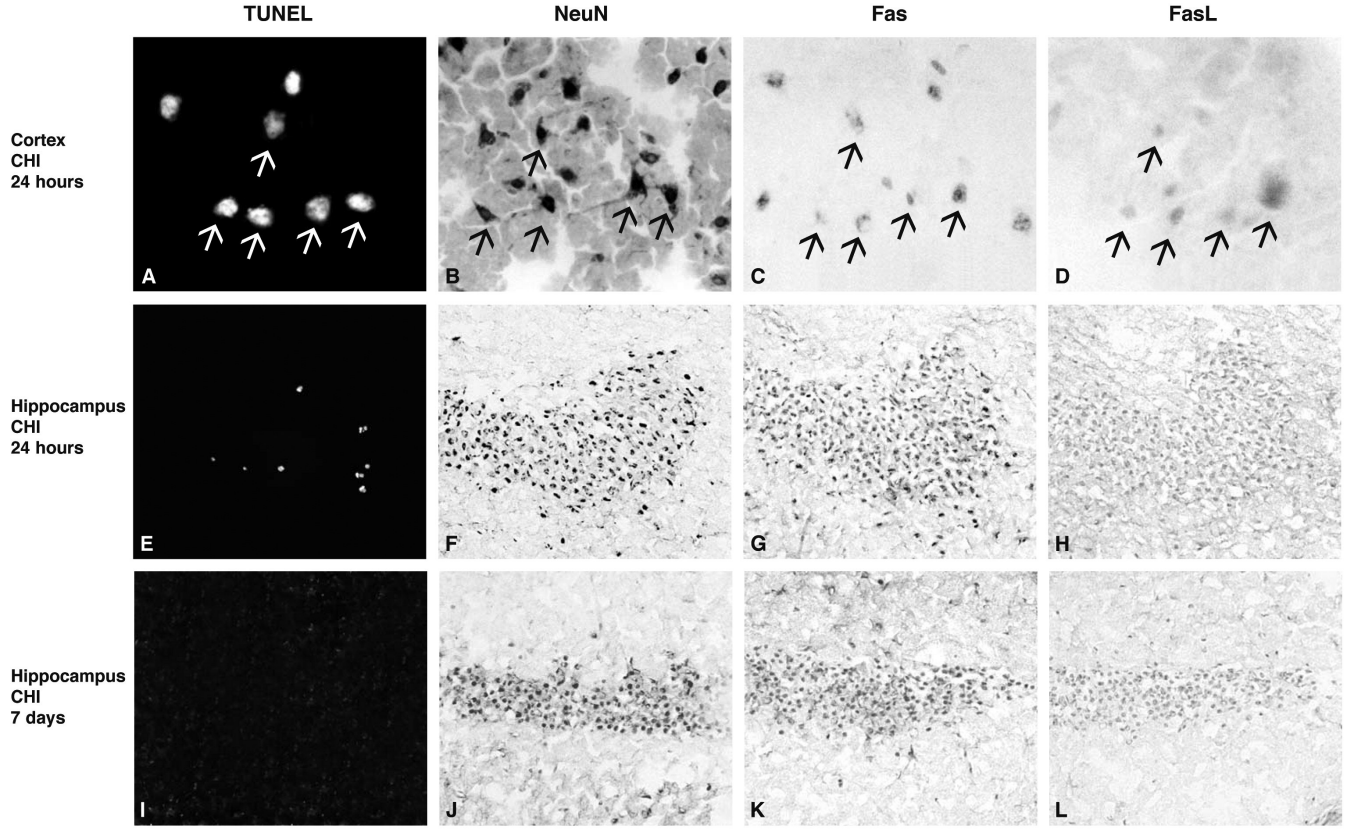

Fig. 4. TUNEL positive cells co-localize with immunoreactivity for neuronal marker NeuN, Fas and FasL. Mice were sacrificed at 24 hours and 7 days after CHI for analysis as described in the "Materials and methods". Photomicrographs show staining for TUNEL (**A, E and I**), NeuN (**B, F and J**), Fas (**C, G and K**) and FasL (**D, H and L**) on consecutive ipsilateral brain sections representatively for B6–/– mice. The first row (**A-D**) shows staining of the cortex at 24 hours after CHI. The second (**E-H**) and third (**I-L**) row show staining of the hippocampus at 24 hours and 7 days post-injury. Most of the cells positive for TUNEL staining were identified as neurons according to the expression of NeuN (arrows, representatively shown in the views for the cortex). These cells also expressed Fas and FasL, as demonstrated by immunohistochemistry with specific antibodies on adjacent brain sections. Only a few hippocampal neurons were labeled by TUNEL staining at 24 hours (**E**), whereas virtually no TUNEL positive cells were found in the hippocampus at 7 days post-injury (I). CHI: closed head injury. A-D, x 1,000; E-L, x 125

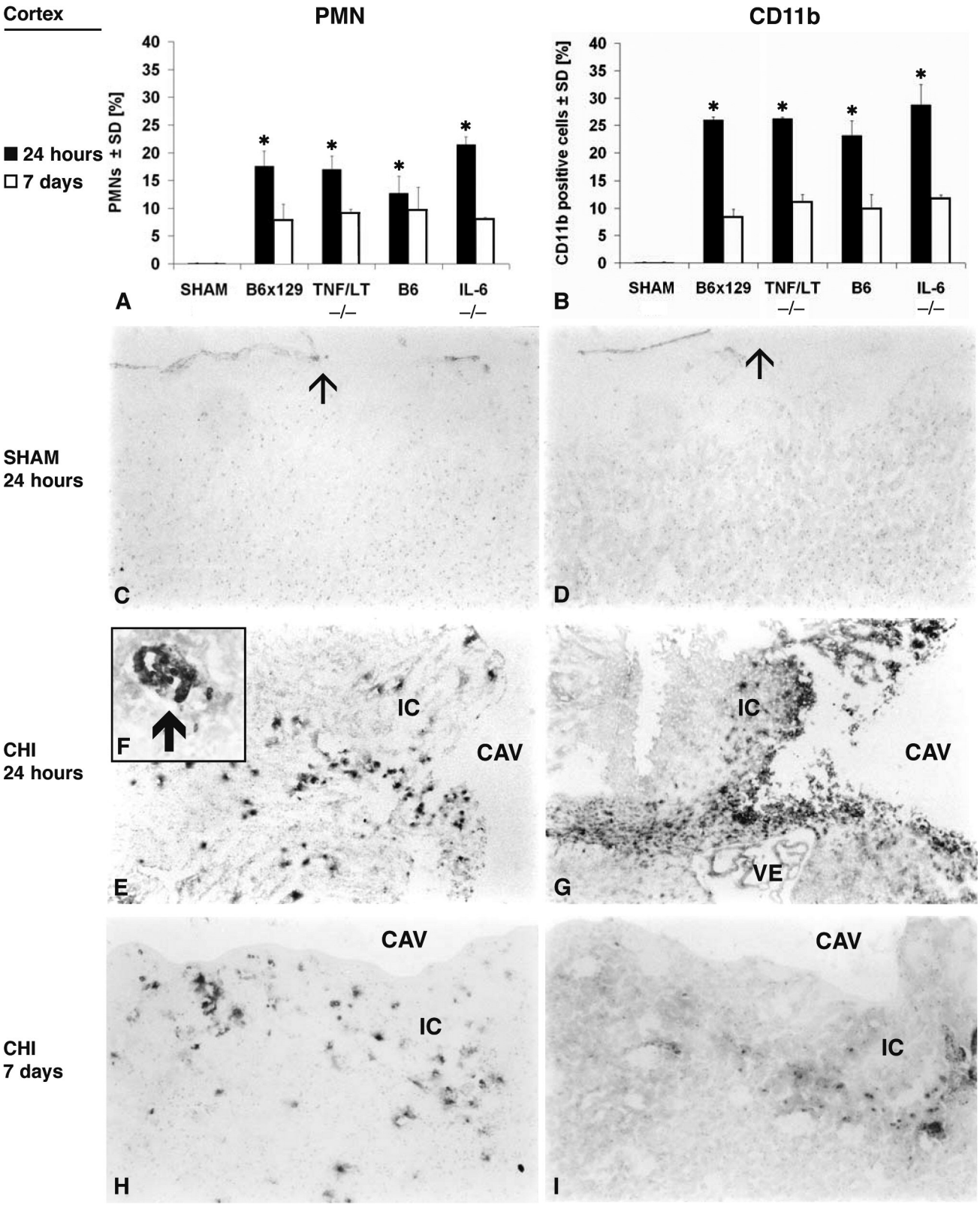

Fig. 5. Accumulated PMNs and macrophages/activated microglia in the injured mouse brain. Serial coronal brain sections of sham-operated mice as well as of mice sacrificed at 24 hours and 7 days after CHI were labeled with anti-PMN (left column) and anti-CD11b (macrophages/activated microglia; right column) antibodies, respectively, together with methyl green counterstaining. Representative photomicrographs of brains of B6–/– mice are shown. The percentages of accumulated PMNs and CD11b positive cells were calculated and their mean values ± SD are shown in the panels A and B. Virtually no PMNs and CD11b positive cells were shown in the brains of sham-operated mice (**A, C and B, D**) whereas a marked accumulation of PMNs and CD11b positive cells was found within the injured hemispheres at 24 hours (**E and G**) and, to a lesser extent, at 7 days (H and I) following CHI. The inset **F** shows the presence of intravascular (thick arrow) and perivascular PMNs. The thin arrows point towards the brain surface. SHAM: sham-operated, IC: injured cortex, CAV: contusion cavity, VE: ventricle, SD: standard deviation, –/–: knockout. *: $P < 0.05$ (24 hours vs. 7 days; Student's t-test). C-E, G-I, x 125; F, x 500

sections as respective markers for neurons, polymorphonuclear leukocytes and macrophages/ activated microglia. TUNEL positive cells were detected in both the injured cortex (Fig. 4A) and ipsilateral CA2/3 hippocampal regions (Fig. 4E). They co-localized mostly with neurons (Fig. 4B,F), as identified by anti-NeuN antibodies, and with Fas (Fig. 4C,G) and FasL expressing cells (Fig. 4D,H) at 24 hours after CHI. At 7 days post-trauma, there were virtually no TUNEL positive cells found in the ipsilateral hippocampus (Fig. 4I). Interestingly, we have discovered a marked overall loss of cells at 7 days post-injury in both the ipsilateral hippocampus (Fig. 4J-L) and injured cortex (data not shown) in the corresponding brain areas which showed extensive cell death by TUNEL staining at 24 hours following CHI. However, it cannot be excluded that some of the TUNEL positive cells may have been leukocytes or microglia. No TUNEL positive cells were found neither in the contralateral hemispheres of the traumatized mice nor in the brains of sham-operated control mice (data not shown).

Post-traumatic cerebral accumulation of PMNs and CD11b positive cells shows only little correlation with TUNEL positive cells

Experimental CHI induced a marked accumulation of PMNs and CD11b positive cells (macrophages and activated microglia) within the injured hemispheres as shown by IHC using anti-PMN and anti-CD11b antibodies (Fig. 5). The percentages of PMNs as well as of CD11b positive cells were calculated for each mouse strain and time point by using light microscopy. In both –/– and wild-type mouse groups, the extent of PMNs and CD11b positive cells was highest in the injured cortex near the site of trauma at 24 hours and was clearly decreased at 7 days post-injury (Fig. 5A,B). These values were generally similar between TNF/LT-α–/– and B6x129 mice, while a marked difference in the percentages of infiltrating PMNs was found at 24 hours when comparing IL-6–/– mice to the corresponding B6 wild-type littermates (21.4%±4.3% vs. 12.7%±2.3%; Fig. 5A). By 7 days after trauma, the percentages of both PMNs and CD11b positive cells in the areas near the impact site did not differ among the genetic different groups but was significantly lower when compared to the corresponding values at 24 hours post-injury (Fig. 5A,B; Student's t-test, $P<0.05$). Only very few PMNs or CD11b positive cells were found in the brains of sham-operated control animals (Fig. 5A-D) and in the contralateral hemispheres of the traumatized mice (data not shown). Apart from a massive accumulation of PMNs and presence of CD11b positive cells in the directly contused cortex, inflammatory cells were also found in the deeper areas of the injured hemispheres at 24 hours after CHI in an uniform distribution pattern in both stainings (Fig. 5E,G). This suggests a strong influence of the mechanisms of secondary brain injury. PMNs were found intravascularly as well as in the proximity of blood vessels, suggesting intrathecal extravasation (Fig. 5F). Furthermore, CD11b positive cells were detected in the ipsilateral corpus callosum at 24 hours post-injury (Fig. 5G). The amount of PMNs and CD11b positive cells decreased but could still be found at 7 days post-trauma (Fig. 5H,I). The distribution of both PMNs and CD11b positive cells showed only little correlation with the localization of TUNEL positive cells as assessed in consecutive brain sections. While PMNs were evenly distributed over a widespread brain area nearby the site of impact (Fig. 5E,H) and most CD11b positive cells were located within the proximate impact area and the ipsilateral corpus callosum (Fig. 5G,I), TUNEL positive cells were found in clearly circumscribed areas, such as in the penumbra of the cortical lesion (Fig. 1C) or in the ipsilateral hypothalamus (Fig. 1E) and co-localized mostly with NeuN immunoreactivity.

Discussion

The model of experimental CHI used in this study produced extensive and prolonged damage in the ipsilateral hemispheres, including abundant DNA degradation, widened contusion cavity and enlarged ventricles, which is consistent with observations previously reported in this model (Shapira et al., 1988; Chen et al., 1996). We found evidence that a large number of TUNEL positive nuclei were present within both the injured cortex and adjacent penumbra zone as well as, to a minor degree, in the ipsilateral hippocampus at the CA2/3 regions. Maximum values for TUNEL positive cells were found within the cortex near the site of impact in the acute phase (24 hours post-trauma) and, but to a lesser degree, at 7 days post-trauma. In the ipsilateral hippocampus, TUNEL positive cells were detected in the CA2/3 regions only at 24 hours post-injury. The nuclei of TUNEL positive cells showed morphological features of both apoptosis and necrosis, supporting the assumption that both mechanisms of cell death occur simultaneously after experimental TBI (Charriaut-Marlangue and Ben-Ari, 1995; Grasl-Kraupp et al., 1995; Kato et al., 1997). Our results suggest that CHI generates a pattern of neuronal cell death in different brain regions similar to that described in previous studies using either this or other models of TBI (Shapira et al., 1993; Pravdenkova et al., 1996; Tang et al., 1997), cortical contusion (Baldwin et al., 1997), controlled cortical impact (Colicos and Dash, 1996; Clark et al., 1997), lateral fluid-percussion brain injury (Rink et al., 1995; Yakovlev et al., 1997; Conti et al., 1998; Knoblach et al., 1999) and cortical cryolesion (Tominaga et al., 1992). It is also important to mention that, at 24 hours following CHI, the percentages of dead cells were slightly increased in both TNF/LT-α–/– mice and corresponding wild-type littermates when compared to IL-6–/– mice and corresponding wild-type littermates, as previously reported by Stahel et al. (2000). A marked neuronal loss was found within the cortex when injured

brains at 24 hours post-trauma were compared to equivalent brain regions of sham-operated control mice. There was also a loss in neurons in the brains of mice sacrificed at 7 days post-trauma when compared to mice sacrificed at 24 hours post-trauma.

In the present study, we identified most of the TUNEL, Fas and FasL positive cells to be neurons according to the co-localizing immunoreactivity with anti-NeuN antibodies. Dying neurons showed the typical morphological features of apoptosis such as chromatin margination and condensation, nuclear shrinkage and formation of apoptotic bodies at both 24 hours and 7 days after experimental CHI. The evident loss of neurons observed in the CA2/3 regions of the ipsilateral hippocampus clearly indicates that this subgroup of neurons is sensitive to Fas-mediated apoptosis at early stages and is likely to degenerate via secondary molecular mechanisms triggered by the primary injury to the nearby cortex. These findings support the evidence that neuronal cell death continues as a chronic process after brain trauma, shifting from the directly contused area into adjoining areas and other proximal regions, as previously reported (Colicos and Dash, 1996; Shohami et al., 1996; Baldwin et al., 1997; Clark et al., 1997; Tang et al., 1997; Conti et al., 1998).

Unlike FasL, constitutive Fas protein expression by cortical and hippocampal neurons of the normal mouse brain was previously described by Park et al. (1998). However, increased Fas and FasL expression was recently demonstrated in studies on experimental and human accidental TBI. Fas and FasL were both localized on cortical neurons and astrocytes of brains of rats for up to 72 hours after injury (Beer et al., 2000). In patients, an elevation of sFas and FasL was detected in the CSF for several days following severe head trauma (Ertel et al., 1997; Lenzlinger et al., 2001). In accordance with other studies, our findings show that there is a marked expression of Fas in the injured cortex and in the ipsilateral hippocampal CA2/3 regions of the mouse brain at 24 hours following experimental CHI. Fas expression was slightly stronger in mice lacking the inflammatory cytokines TNF, LT-α or IL-6 when compared to the respective wild-type littermates. However, this difference was determined by a semi-quantitative analysis and its significance cannot be proven statistically. At 7 days after CHI, Fas remained moderately upregulated in mice of the B6 genetic background (IL-6–/– and wild-type) while it returned to constitutive levels in the B6x129 strain (TNF/LT-α–/– and wild-type). These findings suggest that Fas might be involved in the degeneration of neurons of the injured cortex since its expression coincides with the highest incidence of cell death within the first 24 hours post-injury. CA2/3 hippocampal neurons are also sensitive to Fas-mediated cell death at early stages and are likely to degenerate by apoptosis. Altogether, our findings suggest that Fas expression may be differently regulated depending on the genetic background of the mice. This hypothesis is corroborated by our previous results on the same –/– mice showing the existence of individual susceptibility to blood-brain barrier dysfunction and mortality following experimental CHI (Stahel et al., 2000).

We did not find any evidence of constitutive FasL expression in the brain sections of control mice. However, at 24 hours after experimental CHI, FasL was slightly upregulated in both the cortex near the site of trauma in all mouse strains and the ipsilateral thalamus of IL-6–/– mice and the respective wild-type littermates. FasL was also localized, to a lesser extent, on ipsilateral CA2/3 hippocampal neurons of IL-6–/– mice and the respective wild-type littermates at 24 hours post-injury. A major extent of FasL expression was detected in the injured cortex at 7 days post-trauma when compared to the brains of animals sacrificed at 24 hours post-injury. This data suggests that FasL may be involved in mechanisms of delayed neurodegeneration.

Interestingly, even though Fas and FasL are membrane-associated proteins, the immuno-histochemical staining pattern was not strictly confined to the membrane alone of the positively stained cells. It has been previously shown that Fas and FasL expression can also be found within the cytoplasm of neurons, astrocytes and microglial cells in the injured brains of mice and rats (Martin-Villalba et al., 1999; Beer et al., 2000; Phanithi et al., 2000; Rosenbaum et al., 2000; Qiu et al., 2002; Padosch et al., 2003) as well as in the brains of patients suffering from neurodegenerative diseases (Nishimura et al., 1995; Dowling et al., 1996). The fact that FasL was also present in the cytoplasm corroborates the hypothesis that FasL protein might be stored in the respective cells. This corresponds to previous data showing that FasL-induced apoptosis of Fas-bearing cells is not dependent on de novo synthesis but is due to rapid release of preformed cytoplasmatic FasL in its soluble form (Martinez-Lorenzo et al., 1996; Kiener et al., 1997; Li et al., 1998).

Surprisingly, the maximum upregulation of Fas and FasL protein did not occur simultaneously. It can be assumed that FasL might be the modulating key factor that determines the onset of the apoptotic cascade since a slight increase of its expression within the first 24 hours following injury seems to be sufficient to induce neuronal apoptosis through the binding to Fas receptor expressed at either constitutive or increased levels. It can be presumed that once it reaches maximum concentrations at 24 hours, Fas may be cleaved into the soluble form sFas which suppresses subsequent cell death by competing with the membrane-bound receptor and thus blocking the action of FasL (Cheng et al., 1994). Therefore, the balance between Fas and FasL levels in either the membrane-bound or soluble form seems to be crucial for initiating or suppressing apoptosis.

It is a matter of controversy whether the post-traumatic inflammatory response contributes to secondary brain damage and delayed neurodegeneration. As previously described in various models of TBI,

maximum accumulation of leukocytes was detected within the intracranial lesions at 24 hours post-injury (Biagas et al., 1992; Shapira et al., 1993; Clark et al., 1994; Stahel et al., 2000). In the present study, we show that the accumulation of both PMNs and CD11b positive cells (macrophages and activated microglia) was maximal in the injured hemispheres at 24 hours after trauma in all animal groups and, although diminished, was still increased at 7 days. As we did not find any obvious difference in the accumulation of PMNs or CD11b positive cells between cytokine–/– mice and the corresponding wild-type littermates, we cannot demonstrate an evident role for the cytokines TNF, LT-α or IL-6 in mediating the post-traumatic cerebral leukocyte accumulation. Based on the fact that the leukocyte recruitment preceded the upregulation of FasL, it can be speculated that cell death may be mediated by alternative mechanisms that require further study. To this regard, it has been previously reported that FasL, amongst others, is a potent chemoattractant for neutrophils and may thus sustain their accumulation and the release of other neurotoxic molecules in the injured brain (Ottonello et al., 1999). There is also evidence that both macrophages and microglia play an important role in immunological controlling mechanisms of the central nervous system (Pender and Rist, 2001) and undergo apoptosis in acute inflammatory conditions of the brain such as experimental autoimmune encephalomyelitis (White et al., 1998; Kohji and Matsumoto, 2000). Further studies are required to determine the role of macrophages and microglia in the acute and chronic stages of traumatic neurodegenerative diseases.

In conclusion, the lack of evident differences in the expression of Fas and FasL protein between cytokine–/– and wild-type mice indicates that TNF, LT-α and IL-6 might not play a significant role in the regulation of neuronal death by the Fas/FasL system. However, independent of the impact of cytokines on its expression, our data clearly shows that the Fas/FasL system seems to be involved in neuronal cell death following brain injury. Additional studies need to be undertaken to endorse a conclusive significance of the role of inflammation in neurodegenerative processes occurring within the acute and delayed phases after TBI.

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