Cell-Specific Upregulation of Survivin after Experimental Traumatic Brain Injury in Rats

ERIK A. JOHNSON,1,2 STANISLAV I. SVETLOV,1,2 BRIAN R. PIKE,1,2 PAUL J. TOLENTINO,1,4 GERALD SHAW,1,2,5 KEVIN K.W. WANG,1,2,6 RONALD L. HAYES,1,2,4,6 and JOSE A. PINEDA1,3

ABSTRACT

In this study, we examined the expression and cellular localization of survivin and proliferating cell nuclear antigen (PCNA) after controlled cortical impact traumatic brain injury (TBI) in rats. There was a remarkable and sustained induction of survivin mRNA and protein in the ipsilateral cortex and hippocampus of rats after TBI, peaking at five days post injury. In contrast, both survivin mRNA and protein were virtually undetectable in craniotomy control animals. Concomitantly, expression of PCNA was also significantly enhanced in the ipsilateral cortex and hippocampus of these rats with similar temporal and spatial patterns. Immunohistochemistry revealed that survivin and PCNA were co-expressed in the same cells and had a focal distribution within the injured brain. Further analysis revealed a frequent co-localization of survivin and GFAP, an astrocytic marker, in both the ipsilateral cortex and hippocampus, while a much smaller subset of cells showed co-localization of survivin and NeuN, a mature neuronal marker. Neuronal localization of survivin was observed predominantly in the ipsilateral cortex and contralateral hippocampus after TBI. PCNA protein expression was detected in both astrocytes and neurons of the ipsilateral cortex and hippocampus after TBI. Collectively these data demonstrate that the anti-apoptotic protein survivin, previously characterized in cancer cells, is abundantly expressed in brain tissues of adult rats subjected to TBI. We found survivin expression in both astrocytes and a sub-set of neurons. In addition, the expression of survivin was co-incident with PCNA, a cell cycle protein. This suggests that survivin may be involved in regulation of neural cell proliferative responses after traumatic brain injury.

Key words: astrocyte; neuron; PCNA; survivin; traumatic brain injury

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) is a major health care issue that can lead to permanent motor, cognitive and behavioral deficits. These deficits are the result of neural tissue injury and cell death, most of which occurs within the first days after injury (Raghupathi et al., 2000). The ability of this tissue to resist injury and recover depends

largely on two factors, the survival potential of the cells and the proliferative ability of the cells in the affected area. Therefore, proliferation of cells in response to injury is important in the compensatory/ reparative process. Astrocytes multiply possibly to support surviving neurons and prevent further tissue damage through formation of the glial scar (Ridet et al., 1997; Bush et al., 1999; Smith et al., 2001). Microglia increase to remove cellular debris and promote recovery (Giulian, 1991). Neurons may be replenished by neural stem cells in the dentate gyrus and subventricular zones (Doetsch et al., 1999; Cameron and McKay, 2001; Kernie et al., 2001; Yagita et al., 2001; Peterson, 2002). Consistent with these findings, cell cycle protein expression has been shown after TBI (Kaya et al., 1999a). However, studies have not investigated the role of survivin, a pro-mitotic and anti-apoptotic protein, in the adult brain after TBI.

Survivin is a novel member of the inhibitor of apoptosis protein (IAP) family that can inhibit activated caspases (Ambrosini et al., 1997; LaCasse et al., 1998; Takahashi et al., 1998; Tamm et al., 1998; Deveraux and Reed, 1999; Li and Altieri, 1999; Muchmore et al., 2000; Jiang et al., 2001). Survivin is also an evolutionarily conserved chromosomal passenger protein that is required for proper completion of mitosis. Survivin is present during normal tissue development (Adida et al., 1998; Kobayashi et al., 1999) but is absent in most adult tissues including the brain (Ambrosini et al., 1997; Kobayashi et al., 1999). Many cancer cell lines and cancer tumors, such as neural derived neuroblastoma and glioblastoma, which proliferate at high rates, exhibit survivin over-expression (Altieri et al., 1999; Sasaki et al., 2002). In addition, blocking survivin expression with anti-sense oligonucleotides in these cell lines leads to cell death (Shankar et al., 2001).

In this paper, we demonstrate the induction of survivin expression at the levels of mRNA and protein in the cortex and hippocampus of rats after traumatic brain injury. Survivin protein was primarily localized to astrocytes and in a small subset of neurons as indicated by its co-localization with GFAP and NeuN. In addition, a remarkable induction of proliferating cell nuclear antigen (PCNA) was observed after TBI and also localized to astrocytes and neurons. Finally, survivin and PCNA were co-expressed in single cells suggesting a possible role for survivin in regulation of cellular proliferative responses following TBI.

**MATERIALS AND METHODS**

**Induction of Controlled Cortical Impact Brain Injury**

The surgical and cortical impact injury procedures were conducted as previously described (Dixon et al., 1991; Pike et al., 1998). Briefly, adult male Sprague-Dawley rats (250–300 g) were anesthetized with 4% isoflurane (Halocarbon Laboratories; River Edge, NJ) in 1:1 O2/N2O for 4 min and maintained during surgery with 2.5% isoflurane. Core body temperature was continuously monitored using a rectal thermistor probe and maintained at 36.5–37.5°C using an adjustable heating pad. A unilateral craniotomy (ipsilateral to injury) was performed over the right cortex between the sagittal suture, bregma, and lambda while leaving the dura intact. Traumatic insult was generated by impacting the exposed cortex with a 5-mm-diameter aluminum tip at a velocity of 4 m/sec, a 150-msec dwell time, and a 1.6-mm compression. Craniotomy control animals received the craniotomy but not the impact injury. All procedures were performed according to guidelines established by the University of Florida Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health (NIH).

**Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)**

Survivin primers were generated using GeneBank locus AF 276775: forward primer 5′ TAAGC CACTT GTCCC AGCTT 3′, and reverse primer 5′ GGTTAC CCCAT TACCT 3′, and lambda while leaving the dura intact. Traumatic insult was generated by impacting the exposed cortex with a 5-mm-diameter aluminum tip at a velocity of 4 m/sec, a 150-msec dwell time, and a 1.6-mm compression. Craniotomy control animals received the cra-niotomy but not the impact injury. All procedures were performed according to guidelines established by the University of Florida Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health (NIH).

**FIG. 1.** Survivin mRNA induction in rat brain after traumatic brain injury. Rats were subjected to craniotomy followed by controlled cortical impact brain injury. Total RNA was isolated from injured (ipsilateral) cortex (ic) and hippocampus (ih) at indicated post-injury times. cDNA was synthesized, and quantitative PCR using survivin primers was performed. Data are given as percent of survivin expression over craniotomy controls (C. Cont.); each time point represents mean ± SEM of four independent measurements in craniotomy control or TBI group. **p < 0.01 versus craniotomy control (one-way ANOVA test with post hoc Bonferroni analysis).**
FIG. 2. Expression of survivin protein after TBI in rats. Brain tissue homogenate proteins (40 μg) were separated using SDS-PAGE, immunblotted with survivin antibody, and visualized. (A) Representative Western blot of survivin (17-kDa protein) in ipsilateral cortex (ic) and hippocampus (ih), contralateral cortex (cc) and hippocampus (ch) obtained from injured rats, and from craniotomy control rats without cortical impact (C. Cont.). Densitometry analysis representation of survivin-positive bands in ipsilateral (ic) and contralateral (cc) cortex (B) and ipsilateral (ih) and contralateral (ch) hippocampus (C) after TBI is shown as percent of craniotomy control values. Each data point represents the mean ± SEM of four to six independent experiments. *p < 0.05, **p < 0.001 versus craniotomy control (one-way ANOVA test with post hoc Bonferroni analysis).
gen. Total RNA was isolated from the samples using TRI-zol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA synthesis was performed using 1 μg of total RNA with the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen/Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Q-PCR was performed as previously described (Tolentino et al., 2002) using the LightCycler-FastStart DNA Master SYBR Green I reaction mix (Roche Diagnostics, Indianapolis, IN) in combination with 0.5 μM primers, 2.5 mM MgCl₂ in the Light Cycler rapid thermal cycler system (Roche Diag-

FIG. 3. Expression of PCNA after TBI in rats. PVDF membranes visualized for survivin were stripped and re-probed with PCNA antibody. Representative western blots showing PCNA (36 kDa) (A) and densitometry analysis of PCNA-positive bands (B,C) are presented. Experimental conditions, sample size and abbreviations are identical to those in Figure 2. *p < 0.05, **p < 0.01 versus craniotomy control (one-way ANOVA test with post hoc Bonferroni analysis).
FIG. 4. Immunohistochemistry of survivin and PCNA. Double fluorescent immunostaining for survivin (red) and PCNA (green) was performed in the ipsilateral cortex (A) and hippocampus (B) at 5 day post-injury. Survivin is expressed in the cytoplasm (C, red), while PCNA is expressed in the nucleus (D, green). The white arrow indicates the typical focal co-expression of survivin and PCNA as shown in merged survivin and PCNA images (E). PCNA expression was co-incident with DAPI staining (F, blue, white arrow). Original magnification, ×200; bar = 50 μm (A,B); original magnification ×400, bar = 20 μm (C–F).

Western Blot Analyses

Brain tissue was removed as described above, rinsed with cold PBS, snap frozen in liquid nitrogen and homogenized in ice-cold triple detergent lysis buffer containing a Complete™ protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN). Protein concentration was determined by bicinchoninic acid (BCA) micro protein assays (Pierce, Inc., Rockford, IL). Forty micrograms of protein per well was loaded and separated by SDS-PAGE, transferred to PVDF membranes and probed with goat-anti-rabbit survivin antibody (Novus Biologicals; Littleton, CO; 1:1000). After incubation with goat anti-rabbit HRP-labeled secondary antibody (Biorad, Hercules, CA), the membranes were developed using Enhanced Chemiluminescence Plus reagents (ECL Plus; Amersham, Arlington Heights, IL). For further PCNA analysis, developed PVDF membranes were incubated in stripping buffer, rinsed twice in TBST and incubated with PCNA antibody (Santa Cruz Biotech; Santa Cruz, CA;
Characterization of Survivin Antibody (R51)

First, we compared the specificity of the survivin antibody developed within our group (R51; Dr. G. Shaw) and a commercially available survivin antibody (Chemicon; Temecula, CA). Our antibody showed characteristic staining of the cleavage furrow between dividing HeLa cells consistent with other reports (Li et al., 1998, 1999; Uren et al., 2000). In addition, double labeling with both survivin antibodies showed co-localization at the cleavage furrow. The peptides used to develop our survivin antibody are specific to survivin and do not recognize other IAP family proteins according to SDSC Biology Workbench BLASTP (2.2.2) (Altschul et al., 1997) and CLUSTAL W (1.81) analysis (Higgins et al., 1992; Thompson et al., 1994), resulting in the survivin antibody’s specificity.

Immunohistochemistry (IHC)

Animals were transcardially perfused with 2% Heparin (Elkins-Sinn, Inc.; Cherry Hill, NJ) in 0.9% saline solution (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed in 4% paraformaldehyde and stored in 0.1M PBS or cryo-buffer. Forty micron sections were fluorescent immunolabeled with two primary antibodies in the following experiments: survivin (1:500)/GFAP for astrocytes (Sternberger; Lutherville, MD; 1:1000), survivin/NeuN for mature neurons (Chemicon; Temecula, CA; 1:1000), survivin/PCNA (Santa Cruz Biotech; Santa Cruz, CA; 1:200), PCNA/GFAP and PCNA/NeuN. The nuclear dye DAPI (in Vectashield; H-1200; Vector Laboratories; Burlingame, CA) was used to label the nuclei. The first primary antibody was incubated at 4°C for 24–48 h in a 2% goat serum/2% horse serum/0.2% Triton-X 100 in 0.1 M PBS (block) solution followed by the second primary antibody at 4°C for 1 h in block solution. Fluorescent-tagged secondary antibody (Molecular Probes; Eugene, OR) was used for visualization. For double-labeling using same species antibodies, we used the tyramide signal amplification (TSA) kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer’s instructions and as previously described (Stone et al., 2002). The sections were viewed and digitally captured with a Zeiss Axiosplan 2 microscope (Zeiss, Thornwood, NY) equipped with a SPOT Real Time Slider high-resolution color CCD digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The number of animals used for dual-labeling IHC is as follows: survivin × PCNA = 4, survivin × GFAP = 6, survivin × NeuN = 4, PCNA × GFAP = 4, and PCNA × NeuN = 4. Cell counts were obtained by comparing the number of double-labeled cells to total single-labeled cells in the following groups: survivin/NeuN positive cells to total NeuN positive cells, survivin/PCNA positive cells to total PCNA positive cells, PCNA/NeuN positive cells to total NeuN positive cells, and survivin/GFAP positive cells to total GFAP positive cells. Percentages were calculated by dividing the number of double-labeled cells with the total number of single-labeled cells. For each group, representative photomicrographs were selected and counted. Cells were counted in a total area of at least 188,000 μm² for each group, with no distinction made between cortical and hippocampal regions.

RESULTS

Induction of Survivin Expression after TBI

Q-PCR analysis revealed an initial increase in survivin mRNA at 2 days post injury in the ipsilateral cortex and hippocampus. These transcripts remained elevated in both regions, reached maximum levels at day 5 post-injury and declined at 7 days in the cortex and at 14 days in the hippocampus. All experimental animals remained alive and exhibited slightly impaired motor and cognitive impairments (data not shown). Cortical mRNA levels reached a maximum of 448 ± 10.0%, whereas hippocampal mRNA attained 606 ± 10.0% compared to craniotomy controls (Fig. 2A). In addition, double-labeling with survivin antibodies showed co-localization at the cleavage furrow. The peptides used to develop our survivin antibody are specific to survivin and do not recognize other IAP family proteins according to SDSC Biology Workbench BLASTP (2.2.2) (Altschul et al., 1997) and CLUSTAL W (1.81) analysis (Higgins et al., 1992; Thompson et al., 1994), resulting in the survivin antibody’s specificity.

Immunohistochemistry (IHC)

Animals were transcardially perfused with 2% Heparin (Elkins-Sinn, Inc.; Cherry Hill, NJ) in 0.9% saline solution (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed in 4% paraformaldehyde and stored in 0.1M PBS or cryo-buffer. Forty micron sections were fluorescent immunolabeled with two primary antibodies in the following experiments: survivin (1:500)/GFAP for astrocytes (Sternberger; Lutherville, MD; 1:1000), survivin/NeuN for mature neurons (Chemicon; Temecula, CA; 1:1000), survivin/PCNA (Santa Cruz Biotech; Santa Cruz, CA; 1:200), PCNA/GFAP and PCNA/NeuN. The nuclear dye DAPI (in Vectashield; H-1200; Vector Laboratories; Burlingame, CA) was used to label the nuclei. The first primary antibody was incubated at 4°C for 24–48 h in a 2% goat serum/2% horse serum/0.2% Triton-X 100 in 0.1 M PBS (block) solution followed by the second primary antibody at 4°C for 1 h in block solution. Fluorescent-tagged secondary antibody (Molecular Probes; Eugene, OR) was used for visualization. For double-labeling using same species antibodies, we used the tyramide signal amplification (TSA) kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer’s instructions and as previously described (Stone et al., 2002). The sections were viewed and digitally captured with a Zeiss Axiosplan 2 microscope (Zeiss, Thornwood, NY) equipped with a SPOT Real Time Slider high-resolution color CCD digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The number of animals used for dual-labeling IHC is as follows: survivin × PCNA = 4, survivin × GFAP = 6, survivin × NeuN = 4, PCNA × GFAP = 4, and PCNA × NeuN = 4. Cell counts were obtained by comparing the number of double-labeled cells to total single-labeled cells in the following groups: survivin/NeuN positive cells to total NeuN positive cells, survivin/PCNA positive cells to total PCNA positive cells, PCNA/NeuN positive cells to total NeuN positive cells, and survivin/GFAP positive cells to total GFAP positive cells. Percentages were calculated by dividing the number of double-labeled cells with the total number of single-labeled cells. For each group, representative photomicrographs were selected and counted. Cells were counted in a total area of at least 188,000 μm² for each group, with no distinction made between cortical and hippocampal regions.

RESULTS

Induction of Survivin Expression after TBI

Q-PCR analysis revealed an initial increase in survivin mRNA at 2 days post injury in the ipsilateral cortex and hippocampus. These transcripts remained elevated in both regions, reached maximum levels at day 5 post-injury and declined at 7 days in the cortex and at 14 days in the hippocampus. All experimental animals remained alive and exhibited slightly impaired motor and cognitive impairments (data not shown). Cortical mRNA levels reached a maximum of 448 ± 10.0%, whereas hippocampal mRNA attained 606 ± 10.0% compared to craniotomy controls (Fig. 2A). In addition, double-labeling with survivin antibodies showed co-localization at the cleavage furrow. The peptides used to develop our survivin antibody are specific to survivin and do not recognize other IAP family proteins according to SDSC Biology Workbench BLASTP (2.2.2) (Altschul et al., 1997) and CLUSTAL W (1.81) analysis (Higgins et al., 1992; Thompson et al., 1994), resulting in the survivin antibody’s specificity.

Immunohistochemistry (IHC)

Animals were transcardially perfused with 2% Heparin (Elkins-Sinn, Inc.; Cherry Hill, NJ) in 0.9% saline solution (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed in 4% paraformaldehyde and stored in 0.1M PBS or cryo-buffer. Forty micron sections were fluorescent immunolabeled with two primary antibodies in the following experiments: survivin (1:500)/GFAP for astrocytes (Sternberger; Lutherville, MD; 1:1000), survivin/NeuN for mature neurons (Chemicon; Temecula, CA; 1:1000), survivin/PCNA (Santa Cruz Biotech; Santa Cruz, CA; 1:200), PCNA/GFAP and PCNA/NeuN. The nuclear dye DAPI (in Vectashield; H-1200; Vector Laboratories; Burlingame, CA) was used to label the nuclei. The first primary antibody was incubated at 4°C for 24–48 h in a 2% goat serum/2% horse serum/0.2% Triton-X 100 in 0.1 M PBS (block) solution followed by the second primary antibody at 4°C for 1 h in block solution. Fluorescent-tagged secondary antibody (Molecular Probes; Eugene, OR) was used for visualization. For double-labeling using same species antibodies, we used the tyramide signal amplification (TSA) kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer’s instructions and as previously described (Stone et al., 2002). The sections were viewed and digitally captured with a Zeiss Axiosplan 2 microscope (Zeiss, Thornwood, NY) equipped with a SPOT Real Time Slider high-resolution color CCD digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The number of animals used for dual-labeling IHC is as follows: survivin × PCNA = 4, survivin × GFAP = 6, survivin × NeuN = 4, PCNA × GFAP = 4, and PCNA × NeuN = 4. Cell counts were obtained by comparing the number of double-labeled cells to total single-labeled cells in the following groups: survivin/NeuN positive cells to total NeuN positive cells, survivin/PCNA positive cells to total PCNA positive cells, PCNA/NeuN positive cells to total NeuN positive cells, and survivin/GFAP positive cells to total GFAP positive cells. Percentages were calculated by dividing the number of double-labeled cells with the total number of single-labeled cells. For each group, representative photomicrographs were selected and counted. Cells were counted in a total area of at least 188,000 μm² for each group, with no distinction made between cortical and hippocampal regions.
**FIG. 5.** Co-localization of survivin and GFAP in brain tissue after TBI. Fluorescent immunohistochemistry for survivin (green) and GFAP (red) was performed in the ipsilateral and contralateral cortex (A,B) and in the CA1 and dentate gyrus regions of the hippocampus (G,H) at 5 day post-injury. The injury has completely destroyed the cortex in G leaving only the hippocampus in this picture. Survivin was expressed in the cytoplasm (D,J, green) of GFAP-positive astrocytes (C,I, red) of the ipsilateral cortex and hippocampus and was found to co-localize to these cells as shown in merged C/D and I/J images (E,K, respectively, yellow). White arrows indicate typical survivin-positive astrocytes. Nuclei are shown using DAPI (F,L, blue). Original magnification, ×100, bar = 50 μm (A,B,G,H); ×400, bar = 20 μm (C–F and I–L).

**FIG. 6.** Co-localization of PCNA and GFAP in brain tissue after TBI. Double immunostaining for PCNA (green) and GFAP (red) was performed in the ipsilateral and contralateral cortex (A,B) and the CA1 and dentate gyrus regions of the hippocampus (G,H) at 5 day post-injury. PCNA is present in GFAP-positive cells of ipsilateral cortex (C,D) and, to a lesser extent hippocampus (I,J). (E,K) Merged C/D and I/J, respectively. White arrows indicate typical PCNA-positive astrocytes. PCNA expression was co-incident with DAPI staining (F,L, blue). Original magnification, ×100; bar = 50 μm (A,B,G,H); ×400, bar = 20 μm (C–F, and I–L).
PCNA Expression after TBI

For detection of proliferating cell nuclear antigen (PCNA), PVDF membranes immunostained for survivin were stripped and re-probed using a PCNA-specific antibody. PCNA (36-kDa protein) was significantly detectable in the ipsilateral cortex and hippocampus of TBI rats, but only negligible amounts were observed in the contralateral cortex and hippocampus (Fig. 3A). The temporal patterns exhibited by PCNA protein were similar to that of survivin protein. Namely, PCNA expressed in a time-dependent fashion with a maximum increase at 5 days after injury followed by a gradual decline by 14 days. The levels of PCNA in ipsilateral cortical tissue were raised over craniotomy control by 919 ± 459% at 3 days, 2263 ± 333% at 5 days, and 1035 ± 356% at 7 days post injury (Fig. 3B). Similar increases of PCNA protein in ipsilateral hippocampus were detected at 5 days post injury with a maximum of 1006 ± 229% compared to craniotomy controls (Fig. 3C). No significant increase was found in the contralateral regions when compared to craniotomy controls (Fig. 3A).

Co-Expression of Survivin and PCNA following TBI

To examine spatial co-localization of survivin and PCNA, double-label immunohistochemistry of brain tissue sections was performed on day 5 post injury, when peak expression of these proteins was observed.

At this time point, a remarkable survivin and PCNA immunoreactivity was found in the ipsilateral cortex (Fig. 4A) and ipsilateral hippocampus (Fig. 4B) consistent with data obtained using Western blot analyses. Within both regions, focal co-expression patterns of survivin and PCNA in single cells were detected, which was demonstrated by both separate fluorescent visualization of individual proteins and by merging the images of double-stained slides (Fig. 4C–E). However, the dual expression of survivin and PCNA occurred infrequently as survivin and PCNA immunoreactivity could readily be found separately (Fig. 4C–E). Approximately 12% of the total number of PCNA-positive cells also labeled with survivin. The nuclear morphology of dual survivin and PCNA-positive cells was ambiguous as indicated by DAPI staining (Fig. 4F). Therefore, DAPI staining was simply used for cell identification in all subsequent experiments.

Survivin and PCNA Are Expressed in Astrocytes after TBI

To determine the cell types expressing survivin and PCNA, double-label immunohistochemistry for these proteins and GFAP, a marker of astrocytes, was performed on day 5 post injury.

In accordance with Western blot data, remarkable survivin-positive immunoreactivity was observed in the ipsilateral cortex and hippocampus proximal to the injury cavity (Fig. 5A,G, green) but not in the contralateral areas (Fig. 5B,H). Survivin was co-localized with GFAP in the cells of injured cortex and hippocampus, which strongly suggested primary accumulation of survivin in cells of astrocytic lineage (Fig. 5C–E,I–L). Survivin was uniformly distributed in the cytoplasm and processes of astrocytes in both cortex and hippocampus (Fig. 5D,J). DAPI staining is shown in Figure 5F,L3. Approximately 88% of the total number of GFAP-positive cells also labeled with survivin.

PCNA-positive immunoreactivity staining was observed in the ipsilateral cortex (Fig. 6A, green) and hippocampus (Fig. 6G, green) of injured brain, while contralateral cortex and hippocampus exhibited negligible PCNA immunoreactivity (Fig. 6B,H). PCNA (Fig. 6C,I) was partially co-localized with GFAP (Fig. 6D,J, red) in both regions, and was characteristically distributed in the nucleus of the cells in both cortex and hippocampus (Fig. 6E,K). DAPI staining is shown in Figure 6F,L.

Taken together, double-label immunohistochemistry data provides evidence that both survivin and PCNA can be detected in GFAP-positive astrocytes following traumatic insult. Since survivin and PCNA immunoreactivity was not exclusively localized in GFAP-positive cells, we next addressed a question what other cell type might express survivin after TBI. We suggested that a certain population of mature neurons might express survivin in response to injury.

Survivin and PCNA Are Expressed in a Sub-Set of Neurons after TBI

As can be seen in Figure 7, survivin and PCNA were each co-expressed with NeuN, a marker of mature neurons. NeuN-positive cells were found to express survivin in the ipsilateral cortex distal to the injury cavity (Fig. 7A–D) and in the contralateral hippocampus (Fig. 7E–H). It should be noted, however, that NeuN-positive cells that also expressed survivin occurred infrequently. For example, we estimated the number of dual survivin/NeuN positive cells as 0.1–1.5% of the total number of NeuN-positive cells in these regions. Survivin immunoreactivity was negligible in either hemisphere of craniotomy brains (Fig. 7Q,R). No co-localization of survivin and NeuN was observed in ipsilateral hippocampus (data not shown). As can be seen in Figure 7B,F, survivin was predominantly localized to the cytoplasm and axons of NeuN-positive neurons. DAPI staining is shown in Figure 7D,H.

PCNA-positive neurons were found in the ipsilateral cortex (Fig. 7I–L) and hippocampus after TBI (Fig.
FIG. 7. A sub-set of NeuN-positive neurons express survivin and PCNA after TBI. Double fluorescent immunohistochemistry for survivin (green) and NeuN (red) in the ipsilateral cortex (A,B) and the CA1 pyramidal layer of the contralateral hippocampus (E,F) was performed. Survivin is expressed in the cytoplasm and, to a limited extent, in the processes of NeuN-positive neurons (merged images C/G). Dual staining for PCNA (green) and NeuN (red) is shown in the ipsilateral cortex (I,J) and the CA1 pyramidal layer of the ipsilateral hippocampus (M,N). The nuclei are shown using DAPI staining (D,H, blue). PCNA is expressed in the nucleus of NeuN-positive neurons (merged images K/O). PCNA expression was co-incident with DAPI staining in these examples (L,P, blue). White arrows indicate focal co-localization of survivin/NeuN and PCNA/NeuN. Survivin/NeuN co-localization of survivin (green) and NeuN (red) was seen only in TBI rats as opposed to either hemisphere of craniotomy control (Q,R). Original magnification, ×400, bar = 20 μm (A–P); original magnification, ×50, bar = 100,000 μm (Q,R).
Of particular interest is a sub-set of NeuN-positive neurons found to express survivin only after TBI (Fig. 7). It should be noted that dual staining of survivin and PCNA has been documented previously to express cell cycle proteins in astrocytes and, to a much lesser extent, in neurons in brain cortex and hippocampus. Induction of survivin in these cells was accompanied by occasional expression of PCNA, a cell cycle protein involved in mitotic G1/S progression. Our present data are the first to show that survivin mRNA and protein are significantly up-regulated after traumatic brain injury in rats. PCNA expression after TBI has been described previously (Miyake et al., 1992; Chen et al., 2003), suggesting its role in mechanisms of brain recovery after injury. The concurrent up-regulation of survivin with a similar temporal profile as PCNA shown herein further suggests that survivin may play a role in cellular proliferation after TBI.

Dermal injury evoked the expression of survivin and PCNA in a time-dependent manner (Figs. 2 and 3). Western blot analysis revealed maximal co-expression of both survivin and PCNA at five days post injury. Immunohistochemistry at this time point, demonstrated co-localization of these proteins (Fig. 4), although most cells were labeled separately with PCNA and survivin. In fact, only 12% of the total number of PCNA-positive cells were also survivin positive. It has been reported that PCNA is expressed predominantly in G1/S (Bravo et al., 1987), while survivin is found at the G2/M phase of the cell cycle (Bravo et al., 1987; Otaki et al., 2000). Hence, a lack of strict co-localization of survivin and PCNA in our study may be explained by their expression at different points in the cell cycle.

In our experimental model, we observed survivin- as well as PCNA-positive astrocytes in the proximal area of the injury and in the ipsilateral hippocampus. Proliferation of astrocytes is well documented after TBI as shown by cell labeling with BrdU as well as expression of PCNA (Latov et al., 1979; Dunn-Meynell and Levin, 1997; Carbonell and Grady, 1999; Norton, 1999; Csuka et al., 2000; Kernie et al., 2001; Chen et al., 2003). Because survivin and PCNA were expressed in astrocytes following TBI (Figs. 5 and 6), it is possible that survivin plays an important role linking astrocyte survival and proliferation after traumatic insult. Astrocyte proliferation has been implicated in the formation of the glial scar observed after injury (Latov et al., 1979) and creates a non-permissive environment for repair (Sykova et al., 1999). However, glial proliferation may also enhance neuronal survival (Smith et al., 2001; Wei et al., 2001).

Of particular interest is a sub-set of NeuN-positive neurons found to express survivin only after TBI (Fig. 7). These cells were much less abundant than survivin-positive astrocytes, and their functional significance is currently unknown. However, both neurons and astrocytes have been documented previously to express cell cycle proteins after various insults such as exposure to β-amyloid–activated microglia (Wu et al., 2000), TBI (Kay et al., 1999a,b), chlorine toxicity (Magavi et al., 2000), or as a consequence of Alzheimer’s disease (Yang et al., 2001). These papers underscore the significant controversy that exists regarding the function of cell cycle proteins such as PCNA in neurons after different types of injury. We are currently conducting further studies which will elucidate the roles for PCNA and survivin in neurons after TBI.

It should be noted that dual staining of survivin and PCNA could not be directly attributed to a specific cell type due to the technical difficulties of triple labeling antibody-based IHC. Therefore, we cannot rule out the possibility that other cell types, such as endothelial (Conway et al., 2003) or inflammatory cells (Hill-Felberg et al., 1999), may also contribute to survivin and PCNA expression after TBI. The appearance of survivin and PCNA separately in neurons (NeuN-positive) and astrocytes (GFAP-positive) along with co-localization of survivin with PCNA in the same cells provide correlative data to suggest an activation of cell cycle-like program in astrocytes and possibly in a small subtype of neurons after...
TBI. In our experiments, survivin co-localization with PCNA does suggest that survivin may be associated with a pro-mitotic process. In an attempt to clarify these protein’s roles after TBI, we analyzed the nuclear morphology of survivin-positive cells to define the apoptotic or mitotic architecture of nuclei. DAPI staining proved too ambiguous in identifying apoptotic versus mitotic phenotypes likely due to the thickness of the brain sections (40 µm). Further studies using direct markers of mitosis such as BrdU incorporation as well as simultaneous labeling with cell death related proteins is required to delineate anti-apoptotic and pro-mitotic activities of survivin and PCNA in these cells.

In conclusion, our data demonstrate the induction of survivin in the rat brain following TBI. Expression of survivin occurred predominantly in astrocytes as compared to neurons in a time-dependent fashion and was accompanied by expression of PCNA. Taken together, these results suggest that survivin plays a role in neural cell responses following traumatic brain injury in rats. Future studies will investigate the implications of these findings to the pathophysiology of TBI.

ACKNOWLEDGMENTS

We thank Barbara O’Steen and Tao Fan, M.D., for technical assistance. This work was funded by grants NIH RO1 NS39091, NIH RO1 NS40182, DAMD 17-99-1-9565, and DAMD 17-01-1-0765.

REFERENCES


Address reprint requests to:
Jose A. Pineda, Ph.D.
E.F and W.L. McKnight Brain Institute
University of Florida
100 S Newell Dr., Bldg. 59
LI-118 Dept. of Pediatrics
Gainesville, FL 32610
E-mail: pinedja@peds.ufl.edu