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ORIGINAL ARTICLE

Differential gene expression and alternative splicing of survivin following mouse sciatic nerve injury

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Study design: *In vivo* studies using an axotomy model in adult male Naval Medical Research Institute mice.
Objective: Survivin, a unique member of the inhibitor of the apoptosis (IAP) protein family, is expressed during embryonal development, but is undetectable in terminally differentiated cells and tissues. Owing to the vital role of survivin in cellular proliferation and apoptotic cell death, and also to the necessity of treatment of the nervous system injuries, we have monitored survivin gene expression as well as its alternative splicing changes at different time points within injured mouse sciatic nerves.
Setting: Department of Genetics, School of Basic Sciences, Tarbiat Modares University, Tehran, Iran.
Methods: The sciatic nerves of adult male Naval Medical Research Institute mice were transected and the expression of survivin was examined in the distal and proximal parts of the dissected nerves as well as in the corresponding segments within the spinal cord of the animals, using semi-quantitative RT-PCR and immunohistochemistry.
Results: Survivin is not expressed in undamaged sciatic nerves, but, after sciatic nerve injury, it is gradually upregulated in proximal and distal parts of the dissected nerve ($P<0.05$). Survivin140 is the main variant expressed after injury, accompanied by a low expression of survivin40 ($P<0.05$). There was no expression of the survivin121 variant after injury.
Conclusions: Survivin is differentially expressed and spliced in damaged nerve and spinal cord. Future works on the manipulation of expression and/or the splicing of survivin could decipher the potential effects of survivin variants on the regeneration of nerve and/or spinal cord injuries.
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Keywords: survivin; sciatic nerve; IAP; gene expression; injury

Introduction

During mammalian development, almost half of the initially overproduced neurons are eliminated by apoptosis to define the optimal number of neurons required for normal nervous system function.[1] Deregulation of the balance between cell division and apoptosis results in either tumors or neurodegenerative diseases.

Survivin, a member of the inhibitor of the apoptosis (IAP) family of proteins,[2] is known to be involved in both regulation of apoptosis and control of cell division.[3–5] Survivin is highly expressed during normal tissue development,[6,7] including the nervous system,[7] but it is absent in most terminally differentiated cells of adult tissue.[7,8] This suggests an important role for this protein in cell division. Within the human brain, survivin is expressed in regions populated by neural progenitor cells.[9]

Recent identification of several functionally divergent survivin variants in mouse and human has shown the complexity of survivin action as well as its regulation. The mouse survivin gene has four exons and produces at least three proteins with distinct physiological functions.[10] The largest one, survivin140, is composed of all four exons. This variant contains a single IAP domain and a conserved coiled-coil domain. The IAP domain is found to be antiapoptotic; in a way, it links the biological function of the protein to the cell cycle. The other variant, survivin121, lacks exon 4 and the coiled-coil domain, but retains a part of intron 3. The third variant, survivin40, lacks both IAP and coiled-coil domains and contains only exons 1 and 3, and most likely, it loses its antiapoptotic activity.[10] Different levels of expression for all three variants have been reported during mouse embryonic development.[10,11]

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Furthermore, the cellular functions of survivin can differ depending on the cell type (for example, tumoral vs non-tumoral cells).[4] Owing to the vital role of survivin in cellular proliferation and apoptotic cell death, we have evaluated the alterations in survivin gene expression and its alternative splicing in sciatic nerve injury using a semi-quantitative RT-PCR technique.

Materials and methods

Animal surgery

A total of 24 adult male outbred white Naval Medical Research Institute mice (8–10 weeks old) were obtained from the Razi Institute (Karaj, Iran). For transection of sciatic nerve, the animals were anesthetized with a mixture of xylazine and ketamine (80–100 mg kg^{-1} ketamine + 10 mg kg^{-1} xylazine, i.p.). Briefly, a 3 mm segment of the right-side sciatic nerve was removed by a sharp cut and the distal and proximal stumps of the sciatic nerve were diverted into adjacent muscles to minimize the regrowth of fibers.[12] The apparent health and behaviors of the animals were monitored routinely. At specific time points after the axotomy, the animals were killed by cervical dislocation, and both distal and proximal segments (5 mm of each) of the transected sciatic nerve, intact left sciatic nerve and the lumbosacral part of the spinal cord (L4–L6 segments) were taken for gene expression analysis.

RNA extraction

Total RNA was extracted from frozen tissues using the RNX plus solution (Cinnagen, Tehran, Iran) with some changes to the manufacturer's instructions for a small amount of tissue. Briefly, after homogenizing the tissue, 800 µl of RNX solution and 160 µl of chloroform (Merck kGaA, Darmstadf, Germany) were added to the solution and centrifuged for 15 min at 12 000 *g* at 4 °C. The upper phase was then transferred into another tube and RNA was precipitated with 0.4 ml of isopropanol (Merck) for at least 45 min at 4 °C. The mixture was centrifuged for 15 min at 12 000 *g*, at 4 °C. The resulting pellet was then washed in 75% ethanol and dissolved in diethylpyrocarbonate (DEPC)-treated water.

RT-PCR reaction

Complementary DNA (cDNA) synthesis was carried out using 1 µg RNA and MMLV reverse transcriptase with oligo(dT)$_{18}$ (Fermentas, York, UK) priming in a 20 µl reaction. Specific primers for mouse survivin and β-2 microglobulin (β2m; GenBank accession number: AF115517 and NM-009735, respectively) were used as described earlier.[11]

The sequences of the primers used were as follows:

msurvivin forward: 5′-TCGCCACCTTCAAGAACTGGCC CTTCCTGGA-3′

msurvivin reverse 1: 5′-GTTTCAAGAATTCACTGACGGT TAGTTCTT-3′

msurvivin reverse 2: 5′-GGCTTCTGACAATGCTTG-3′

mβ2m forward: 5′-TGACCGGCTTGTATGCTATC-3′

mβ2m reverse: 5′-CACATGTCTCGATCCCAGTAG-3′

The primers were synthesized by MWG Biotech Company (Ebersberg, Germany) as highly purified salt-free grade. All primers used were blasted against the mouse genome to ensure that they are not complementary with other regions of the genome.

PCR primers were expected to amplify 254, 144, 332 and 316 bp segments from survivin140, survivin40, survivin121 and β2m cDNA, respectively. PCR (Techne, Cambridge, UK) was carried out using 2 µl of synthesized cDNA with one unit of Taq polymerase (Cinnagen). The PCR amplification was carried out for either 35 (survivin), 30 (β2m in nerve) or 26 (β2m in spinal cord) cycles. The cycling conditions were as follows: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, with a final extension at 72 °C for 10 min. A no-RT control was carried out in each set of PCR.

Quantification of the intensity of PCR products

PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. The amount of DNA was quantified by measuring the intensity of light emitted from corresponding bands under UV light using Labimage software (version 2.6; Kapelan GmbH Co., Halle, Germany).

The results were expressed as the ratio of the intensity of the survivin gene band to that of β2m as an internal control,[11]wherein the comparisons were made for matched internal controls.

Statistical analysis

All experiments were replicated at least thrice and the results were analyzed by performing analysis of variance (ANOVA) followed by Tukey tests, with $P<0.05$ considered as statistically significant. The s.d. was also calculated for each value.

Immunohistochemistry

Tissue samples from four injured and one un-injured mice were collected. 5 µm tissue sections were deparaffinized in xylene and rehydrated by passing through graded alcohols. For antigen retrieval, slides were placed in a water bath with 9 mM sodium citrate, pH = 6, for 15 min in 95 °C and then gently cooled and washed with phosphate buffered saline. Endogenous peroxidase activity was suppressed with 1.5% H_2O_2 for 20 min. The slides were serum-blocked (with 10% normal goat serum) and incubated with a polyclonal antibody against survivin (1:1000, Novous Biologicals, Littleton, CO, USA) at 4 °C for 24 h. The sections were then incubated overnight with a secondary antibody (1:250, biotinylated anti-rabbit IgG, Vector Loboratories, Burlingame, CA, USA) at 4 °C. The sections were then incubated for 2 h with Avidin/HRP (1:250, Dako, Glostrup, Denmark) at room temperature. The slides were rinsed with phosphate buffered saline and immersed in a freshly prepared DAB/H_2O_2 for 15 min at room temperature.

Results

RT-PCR optimization

RT-PCR reactions were optimized by varying the number of amplification cycles, in increments of two cycles, starting

from 20 to 40 cycles, to select the lowest cycle number in which transcripts could be quantified without reaching the stationary phase. The band intensity increased almost linearly as the reaction approached the stationary phase. An amplified band corresponding to the expected size for the β2m gene appeared at cycle 26 for spinal cord and at cycle 30 for sciatic nerve samples (data not shown). The same procedure was used for the amplification of survivin, and the cycle number of 35 was used for all PCR reactions (data not shown).

The size of the amplified DNA fragment for both survivin140 (254 bp) and survivin40 (144 bp) was as expected for both variants (Figure 1). To further confirm the accuracy of the PCR products, the amplified survivin and β2m products were digested with an *Eco*RI restriction enzyme. This generated two smaller fragments for both survivin140 (242 + 12 bp) and survivin40 (132 + 12 bp) variants, as well as two smaller fragments for β2m (222 + 94 bp). These sizes were, as expected, based on the calculated products from the primary sequences of survivin and β2m (data not shown).

Changes in the expression of survivin variants after nerve injury

Four different tissue samples were collected and analyzed as the following: distal and proximal segments of transected sciatic nerve, intact sciatic nerve on the other side (as control), and L4–L6 segments of the spinal cord (L4–L6 segments of un-injured animal used as a control).

The gene expression levels were examined for survivin variants using a semi-quantitative RT-PCR technique. To ensure that equal amounts of RNA were used for each reaction and that differences in the signal intensity were not because of the differences in the amounts of starting RNA, β2m was used as an internal control for each amplification reaction. RT-PCR was carried out in separate tubes under similar conditions (except for the cycle number) for both survivin and β2m with results expressed as survivin/β2m expression ratio. All experiments were repeated at least thrice, and the amplified products were loaded onto agarose gel and electrophoresed. As expected, β2m was expressed in all specimens. The PCR for survivin produced two DNA segments of 254 and 144 bp in specimens obtained from distal (Figure 1a) and proximal (Figure 1b) segments of dissected sciatic nerve, and only a 254 bp single band in the spinal cord (Figure 1c).

There was a significant variation in the level of expression, both in different variants as well as in different samples, obtained from the sciatic nerve and the spinal cord. Both survivin140 and survivin40 were absent in undamaged sciatic nerve, but they were gradually expressed in distal and proximal segments of the sciatic nerve with different intensity, in which survivin140 was the prominent variant (Figures 1 and 2). In spinal cord segments, there was no detectable expression of survivin40. However, the expression of survivin140 was evident in the spinal cord of both the control and nerve injury model (Figures 1c and 2e). There

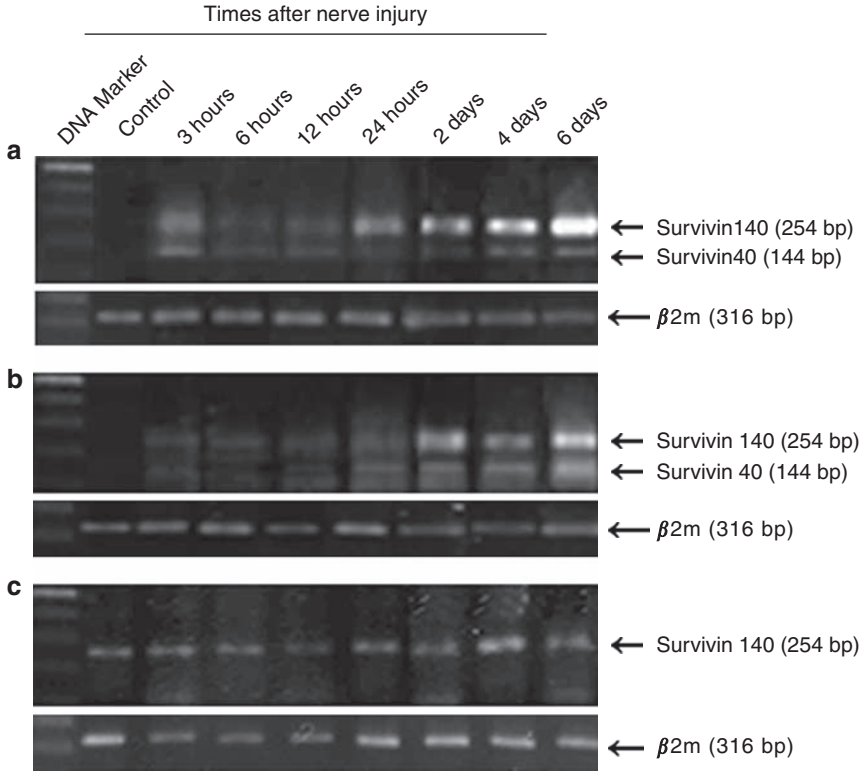

Figure 1 RT-PCR analysis of survivin gene expression in distal (**a**) and proximal (**b**) segments of dissected sciatic nerve and its corresponding (L4–L6) segments of the spinal cord (**c**) before and at specific time points after injury. Note that there is no expression of survivin in control (the uncut/left sciatic nerve from the same animals) nerve samples and that there is no expression of survivin40 in spinal cord samples (the spinal cord's control was from an uninjured animal). The lower band in each lane represents the RT-PCR products for β2m (internal control).

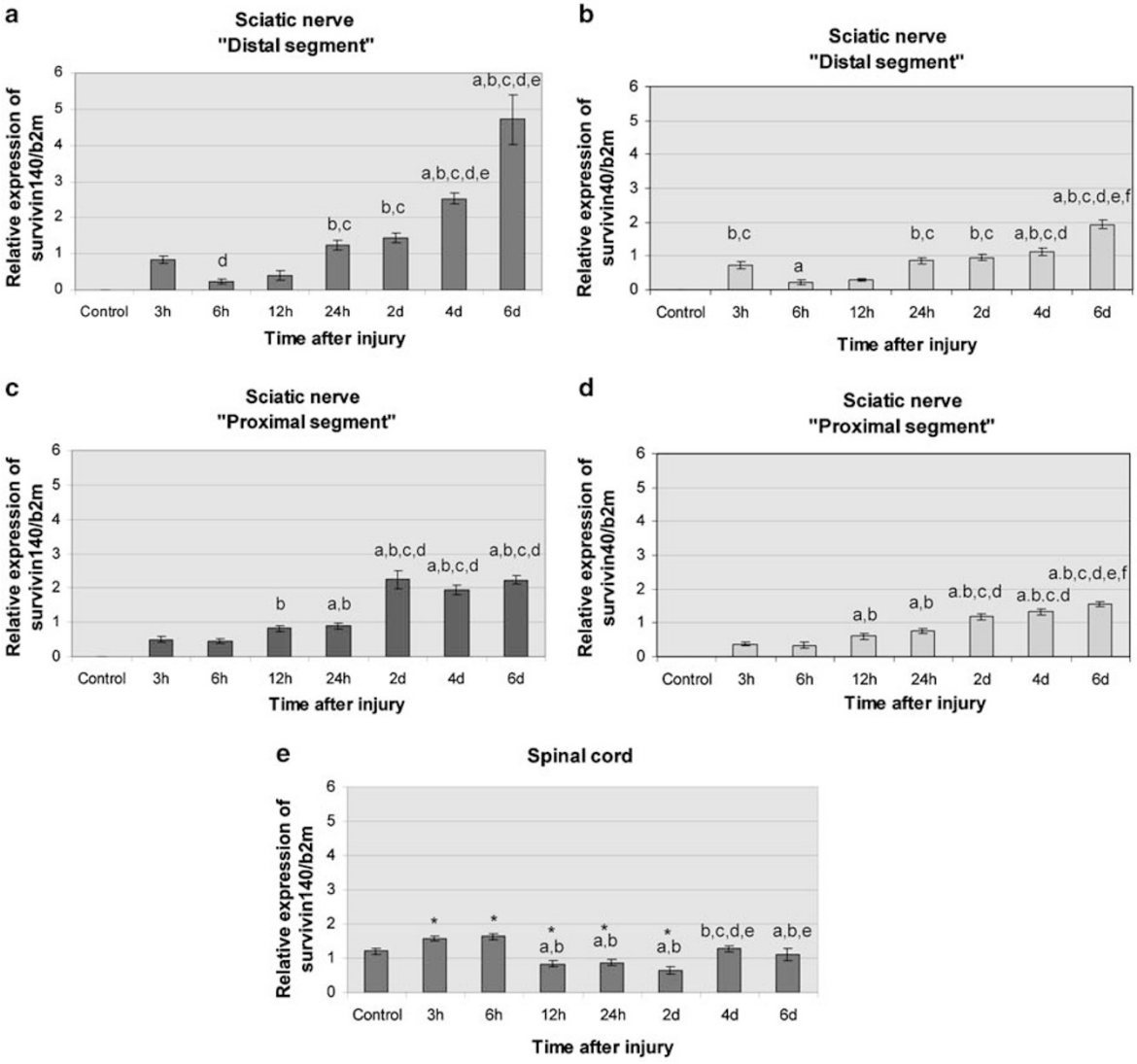

Figure 2 Histograms comparing the relative expression of survivin variants in control (undamaged animal) and damaged nerve as well as spinal cord samples at different time points after sciatic nerve injury in (**a**) Survivin140 in the distal segment of the dissected sciatic nerve. (**b**) Survivin40 in the distal segment of the dissected sciatic nerve. (**c**) Survivin140 in the proximal segment of the dissected sciatic nerve. (**d**) Survivin40 in the proximal segment of the dissected sciatic nerve. (**e**) Survivin140 in L4–L6 segments of the spinal cord. Values are shown as the mean ± s.d. (Significant difference with: * = control, *a* = 3 h, *b* = 6 h, *c* = 12 h, *d* = 24 h, *e* = 2 d, *f* = 4 d and *g* = 6 d groups).

was no detection of survivin121 variant in the proximal and distal segments of the sciatic nerve or in their corresponding segments in the spinal cord.

The mean for the relative expression of survivin variants was normalized to the expression of β2m and the significance of the differences was tested by ANOVA (Figure 2).

Immunohistochemistry of survivin protein

To determine the cellular expression of survivin at the protein level, we used the immunohistochemistry technique using a specific antibody against survivin, which detects all variants of survivin. After optimization of the method, the expression of the survivin protein was evaluated on the sciatic nerve segments and on the L4–L6 segments of the spinal cord after 6 days (144 h) of axotomy. The results showed both nuclear and cytoplasmic distribution of the survivin protein in the spinal cord (Figure 3a), but there was a very weak or no signal of survivin in either distal or proximal segments of the sciatic nerve (Figure 3c). The immunoreactivity was seemingly distributed within both dorsal and ventral horns but not within the white matter. There was also no noticeable difference between the signal strength of survivin between damaged and un-damaged samples. There was no signal in the control sections in which all the conditions were maintained the same, except for omission of the first antibody (Figures 3b, d).

Discussion

A hallmark of many neurodegenerative diseases is neuronal cell death.[13] In apoptotic cell death, neurons are actively

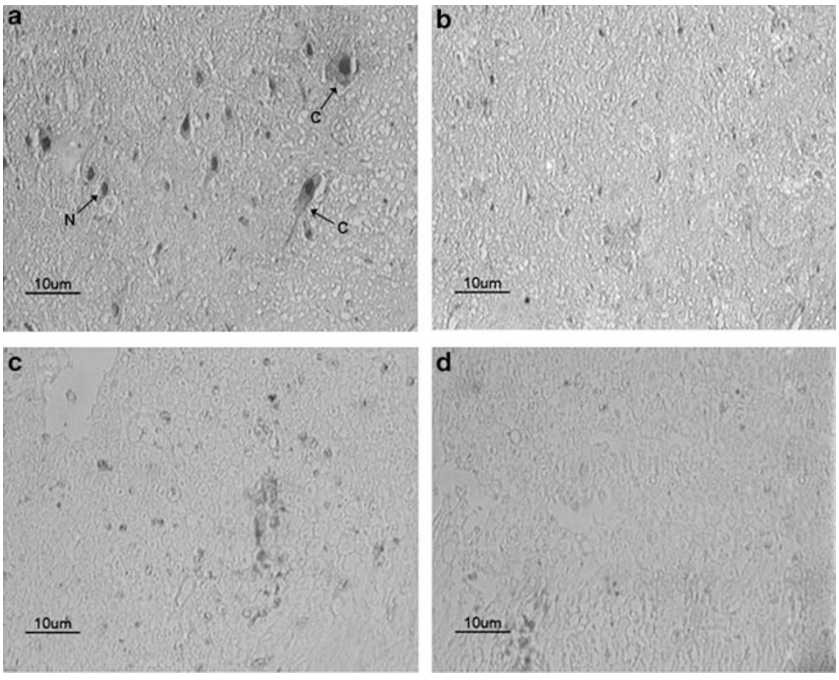

Figure 3 Immunohistochemistry of L4–L6 segments of the spinal cord and sciatic nerve at 6 days (144 h) after sciatic nerve injury. (**a**) Nuclear and cytoplasmic distribution of survivin in spinal cord segments. The signal was seemingly distributed within both dorsal and ventral horns but not in white matter. (**b**) Negative control of spinal cord. All the conditions are similar to A, except for the omission of the first antibody. (**c**) No or very weak signal of survivin protein in injured proximal (the same observation for distal) sciatic nerve. (**d**) Negative control of sciatic nerve segments. All the conditions are similar to C, except for the omission of the first antibody.

responsible for their own death by switching on/off a number of cell-death regulatory genes or intracellular pathways.[1,14] Given that the central nervous system has very limited regenerative capacity, it is of utmost importance to limit the damage caused by neuronal death.[15]

As a member of the IAP family, survivin plays a crucial role in both cell proliferation and apoptosis.[8] Furthermore, alternatively spliced variants of the gene seem to have their own unique subcellular localizations and (sometimes opposing) functions.[10] Although the overexpression of survivin is widely studied in various tumors, including glioblastomas, meningiomas, schwannomas and neurofibromas,[16] very little attention has been given so far to its expression profile in neurodegenerative disorders.

Here, we have evaluated the alterations in the expression of different variants of survivin after axotomy of the mouse sciatic nerve. Our data indicate a differential expression of survivin variants in the proximal and distal parts of the dissected nerve as well as in their corresponding neuronal cell bodies located within L4–L6 segments of the spinal cord. Our data showed for the first time that (1) there is no detectable expression of survivin variants in undamaged sciatic nerve, whereas there is some expression in the spinal cord. (2) Both survivin140 and survivin40 variants are absent in the sciatic nerve, but they are gradually overexpressed in the proximal and distal parts of the damaged nerve, where they reach their peak of expression, within proximal segments, 2 days after the damage. This observation is consistent with earlier works in which significant gene/protein expression alterations were reported 3 days after axonal injury. [17,18] A possibility for such a delay in reaching the peak of gene expression alterations might be the induction of glial cells (for example, Schwann cells) and macrophage proliferation 2 days after postaxotomy.[19] These proliferative cells could be the cause for the the observed overexpression of survivin after nerve injury.

The notion that there is no survivin140 expression in undamaged sciatic nerve and that there is no expression of survivin40 in undamaged and damaged spinal cord could be eluding to the differential expression and alternative splicing of survivin within the central and peripheral nervous system. The difference is probably established through different microenvironments and/or different cell type compositions of the spinal cord and sciatic nerve. In other words, the survivin variants could have different roles within the damaged nerve. To find out whether the overexpression of the survivin variants has a causative effect in the process of regeneration, or whether it is simply a byproduct of the global changes on the damaged nerves, would require further investigation. Performing the same experiment on a transgenic mouse model in which the expression of survivin is knocked out in neuronal[15] or glial cells would provide a clear answer to the question. (3) Although our designed primers are adequate to detect survivin21,[10,11] no expression of this variant in damaged/undamaged samples of either sciatic nerve or spinal cord was observed. This finding is consistent with the earlier report by Emadi *et al*.,[11] in which they have reported the lack of expression of survivin121 during early brain development of mouse brain. The finding, however, is in contrast to the results of

Conway[10] that reported the expression of this variant in most already-developed adult tissues of mice. The inconsistency might be because of the use of different tissue samples. Emadi *et al.*,[11] had worked on a fetal as well as a postnatal brain model, in which survivin likely plays a vastly different role than in the already-developed adult brain tissue. (4) Survivin140 is the prominent variant in the damaged nerve and the only variant expressed in the spinal cord. This is again in agreement with the same observation by Emadi *et al.*,[11] who reported that survivin140 appeared to be the major variant in developing mouse brain. The biological property of the survivin40 variant during neurogenesis and central nervous system development is unclear and needs further investigation. As survivin40 has retained the N-terminal domain, it may form dimers with other variants and modulate their function.[10] (5) Using immunohistochemistry, we confirmed the expression of survivin in injured spinal cord and sciatic nerves at the protein level. As the antibody detects all variants of survivin, it is not feasible to compare the immunohistochemistry results with those of RT-PCR. Furthermore, a possible differential post-transcriptional mechanism of gene expression in spinal cord versus sciatic nerve could be the reason for the observed inconsistency between our RT-PCR and immunohistochemistry results. Moreover, further works are needed to find out the types of cells that are producing survivin after injury. In peripheral nerve injury, survivin could be produced by Schwann cells or inflammatory cells that are recruited to the injury site.[19] Moreover, within the injured spinal cord, survivin could be synthesized by any number of different types of cells, including astrocytes, macrophages/microglia and oligodendrocytes.

The expression of survivin is extensively studied by Jiang *et al*.[15] and by Emadi *et al*.[11] during early brain development. Furthermore, the expression was also studied after traumatic brain injury by Johnson *et al*.[20] On the basis of their report, the expression of survivin was time dependent and cell specific, and was present in astrocytes and, to a much lesser extent, in neurons of 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.[20] Our presented data, along with earlier findings, provide a clearer picture on the involvement of survivin in normal and abnormal physiology of the nervous system.

Conclusion

In conclusion, our data suggest that survivin is differentially expressed and spliced after nerve injury. However, more work is needed to elucidate the complex role of survivin variants in neuronal and glial cells.

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