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

Article *in* Spinal Cord · April 2009

DOI: 10.1038/sc.2009.26 · Source: PubMed

Differential gene expression and alternative splicing of survivin during regeneration of injured mouse sciatic nerves

Running title: Survivin expression in injured mouse sciatic nerves

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Abstract:

Study design: *in vivo* study using an axotomy model in adult male NMRI mice.

Objective: Survivin, a unique member of the inhibitor of apoptosis (IAP) protein family, is expressed during embryonal development but is undetectable in terminally differentiated cells and tissues. Because of the vital role of survivin in cellular proliferation and apoptotic cell death, and also the necessity of the treatment of the nervous system injuries, we have monitored survivin gene expression as well as its alternative splicing changes during the course of regeneration in injured mouse sciatic nerves.

Setting: Department of Genetics, School of Basic Sciences, Tarbiat Modares University, Tehran, Iran

Methods: Sciatic nerve of adult male NMRI mice were transected and the expression of survivin variants were determined in distal and proximal parts of the dissected nerve as well as the corresponding segments within spinal cord of the animals, with semi-quantitative RT-PCR technique.

Results: Survivin is not expressed in undamaged sciatic nerve, but after sciatic nerve injury is gradually upregulated in proximal and distal parts of the dissected nerve. Survivin140 is the main variant expressed after injury, accompanied by low expression of survivin40. There was no expression of survivin121 variant after the injury.

Conclusions: Survivin is differentially expressed and spliced during the course of regeneration in damaged nerve and spinal cord. The obtained data suggest that manipulation of expression and/or splicing of survivin could potentially affect the process of regeneration in nerve and/or spinal cord injuries.

Keywords: survivin; sciatic nerve; IAP; spinal cord; gene expression; injury

Introduction:

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

Survivin, a member of the inhibitor of apoptosis (IAP) family proteins[2-4] is known to be involved in both regulation of apoptosis and control of cell division.[5-8] It is highly expressed during normal tissue development[9,10], including the nervous system,[10] but is absent in most terminally differentiated cells of adult tissues.[10,11] suggesting an important role for this protein in cell division. Within the human brain, it is expressed in regions populated by neural progenitor cells.[12]

Recent identification of several functionally divergent survivin variants in mouse and human increases the complexity of survivin action as well as its regulation. Mouse survivin gene has 4 exons, and produces three proteins with distinct physiological functions.[13] The largest one, ($Survivin_{140}$) results from translation of all four exons. This variant contains a single IAP domain, which is responsible for its anti-apoptotic property, and a coiled-coil domain, which links biological function of the protein to the cell cycle. The other variant ($survivin_{121}$) lack exon 4, coiled-coil

domain, but contains a part of intron 3. The third variant ($Survivin_{40}$) lack both IAP and coiled-coil domains and has only exons 1 and 3; and probably has lost its anti-apoptotic activity. Different levels of expression for all three variants were reported during mouse embryonic development[13]. Furthermore, the cellular functions of survivin can vary depending on the cell type and cellular context studied.

Due to the vital role of survivin in cellular proliferation and apoptotic cell death, we have evaluated the alterations in survivin gene expression and alternative splicing during the course of regeneration in injured sciatic nerves using semi-quantitative RT-PCR technique.

Materials and Methods:

Animal surgery:

A total of 24 adult male NMRI mice were obtained from Pasteur Institute (Karaj, Iran). For transection of sciatic nerve, animals (30-40 g body weight) were anesthetized with a mixture of xylazine and ketamine (80-100 mg/kg ketamine+10mg/kg xylazine, i.p.). Briefly, a 3 mm segment of the right-side sciatic nerve was removed and the distal and proximal stump of the sciatic nerve was diverted into adjacent muscles in order to minimize regrowth of the fibers. For gene expression analysis, at specific time points (3, 6, 12, 24, 48, 96 and 144 hours) after the axotomy, the animals were sacrificed 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 removed. All dissected tissues were immediately frozen on liquid nitrogen and stored at -80ºC before being used for RNA extraction.

RNA extraction

Total RNA was extracted from frozen tissues using the RNX plus solution (Cinnagen, Iran) with some changes to the manufacturer's instructions for small amount of tissue. Briefly, after homogenizing the tissue, 800 µl of RNX solution was added to the tube and then 160 µl of chloroform was added to the solution and centrifuged for 15 min at 12000 g at 4ºC. The

upper phase was then transferred to another tube and RNA precipitated with 0.4 ml of isopropanol for at least 45 minutes at 4ºC. The mixture was centrifuged for 15 min at 12000 g at 4ºC and the resulting pellet was then washed in 75% ethanol and dissolved in DEPC-treated water.

The purity and integrity of the extracted RNA was evaluated by optical density measurements (260/280 nm ratios) and by visual observation of samples electrophoresed on agarose gels. Both methods confirmed the integrity of the extracted RNA with little or no protein contamination.

RT-PCR Reaction

Complementary DNA synthesis reaction were performed using 1μg RNA and MMLV reverse transcriptase with oligo$(dT)_{18}$ (Fermentas) priming in a 20 μl reaction as described elsewhere.[14] Specific primers for mouse survivin and beta-2microglobulin (β2m) as an internal control, GenBank accession number: AF115517 and NM-009735 respectively, were used as described previously.[15,13]

The sequences of the employed primers were as follows:

msurvivin forward: 5' TCGCCACCTTCAAGAACTGGCCCTTCCTGGA3'

msurvivin reverse 1: 5'GTTTCAAGAATTCACTGACGGTTAGTTCTT 3'

msurvivin reverse 2: 5' GGC TTC TGA CAA TGC TTG 3'

mβ2m forward: 5' TGA CCG GCT TGT ATG CTA TC 3'

mβ2m reverse: 5' CAC ATG TCT CGA TCC CAG TAG 3'

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

PCR primers amplified 254, 144 and 316 bp segments from Survivin140, Survivin40 and β2m cDNA respectively. PCR was performed using 2 μl of synthesized cDNA with 1 unit of Taq polymerase (Cinnagen, Iran). The PCR amplification was performed for either 35 (survivin), 30 (β2m in nerve) or 26 (β2m in spinal cord) cycles. The cycling conditions were as follows: 94 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 40 s with a final extension at 72 ºC for 10 min.

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., Germany). The results were expressed as the ratio of the intensity of the survivin gene band to that of β2m to account for any differences in the starting amounts of RNA.

Statistical analysis

All experiments were replicated at least three times and the results were analyzed by performing Tukey-tests with $P<0.05$ considered as statistically significant. The S.D. was also calculated for each value.

"We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research".

Results:

RT-PCR optimization

The RT-PCR reaction was optimized by varying the number of amplification cycles, in increments of 2, from 20 to 40 cycles, in order to select the lowest cycle number where transcripts could be quantified without reaching stationary phase. Band intensity increased almost linearly as the reaction approached the stationary phase. A band corresponding to the expected size for β2m gene appeared at cycles 26 for spinal cord and cycles 30 for sciatic nerve samples (data not shown). The same procedure was employed for survivin, and the cycle number of 35 was used for all PCR reactions of survivin of specimens before and after different time points of injury (data not shown).

The sizes of the amplified DNA fragments for Survivin140 (254 bp) and Survivin40 (144 bp) were as expected for both variants (Fig. 1). To further confirm the accuracy of the PCR products, the amplified products were digested with *Eco*RI restriction enzyme. Digestion generated two smaller fragments for both variants, with sizes as expected from the primary sequences (data not shown).

Changes in the expression of survivin variants after nerve injury

To determine the changes in the expression and alternative splicing of the survivin gene during the course of nerve regeneration, four different tissue

samples were collected and analyzed: distal and proximal segments of transected sciatic nerve, intact sciatic nerve on the other side (as control) and the L4-L6 segments of spinal cord.

Gene expression levels were examined by semi-quantitative RT-PCR technique. To ensure that equal amounts of RNA were used for each reaction and differences in the signal intensity were not due to the differences in the amounts of starting RNA, β2m was used as an internal control for each reaction. RT-PCR was performed 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 three times and the amplified products were loaded onto agarose gel and electrophoresed. β2m was expressed in all specimens and the PCR products for survivin produced two DNA segments of 254 bp and 144 bp in specimens obtained from distal (Fig 1A) and proximal (Fig 1B) segments of dissected sciatic nerve and only a 254 bp single band in the spinal cord (Fig 1C).

There was a significant variation in the level of expression both in different variants as well as different samples obtained from sciatic nerve and spinal cord. Both survivin140 and survivin40 was absent in undamaged sciatic nerve, but gradually expressed in distal and proximal segments of sciatic nerve with different intensity, where the survivin140 was the prominent variant (Fig 1 and 2). In spinal cord segments, there was no

detectable expression of Survivin40. In contrast, the expression of survivin140 was evident in spinal cord of both control and nerve injury model (Fig 1C, 2C)

There was no detection of survivin121 variant neither in proximal and distal segments of sciatic nerve nor in their corresponding segments in the spinal cord.

Discussion:

A characteristic of many neurodegenerative diseases is neuronal cell death.[16] Among the modalities by which neurons die, apoptosis has a very unique place, because in this type of cell death neurons are actively responsible for their own death by switching on/off a number of cell-death regulatory genes or intracellular pathways.[1] Given that central nervous system tissue has very limited, if any, regenerative capacity, it is of utmost importance to limit the damage caused by neuronal death.[17]

As a member of IAP family, survivin plays an important role in both cell proliferation and apoptosis.[11] Furthermore, alternatively-spliced variants of the gene appear to have their own unique subcellular localizations and (sometimes opposing) functions.[13] While the overexpression of survivin are widely studied in various tumors, including glioblastomas, meningiomas, schwannomas and neurofibromas,[18] very little attention has been received so far to its expression changes in neurodegenerative disorders.

Here, we have evaluated the alterations in the expression of different variants of survivin following the axotomy of mouse sciatic nerve. To detect potential variants of survivin in mouse neural tissues, two reverse primers were designed on different exons, while forward primer was on the first exon. Our data reveals a differential expression of survivin variants in proximal and distal parts of dissected nerve as well as their corresponding neuronal cell bodies located within L4-L6 segments of spinal cord. Our data

revealed for the first time that: 1) there is no detectable expression of survivin variants in undamaged sciatic nerve, while there is some expression in the spinal cord. 2) Both survivin140 and survivin40 variants are absent in sciatic nerve but gradually overexpressed in proximal and distal parts of the damaged nerve, where they reach their pick of expression 2 days after the damage. This finding suggests that these variants might have potential role during the regeneration of damaged nerve. To find out whether this overexpression has a causative effect in the process of regeneration or it is simply a byproduct of the global changes in the damaged nerves requires further investigation. Performing the same experiment on a transgenic mouse model in which the expression of survivin is knocked out in neuronal[17] or glial cells would provide a clear answer to the question. 3) There is no detectable expression of survivin121 variant in damaged/undamaged samples of neither sciatic nerve nor spinal cord. This finding is consistent with the previous report by Emadi et al, in which they report the lack of expression of survivin121 during both fetal and postnatal development of mouse brain.[19] The finding, however, is in contrast with the Conway's results that reported the expression of this variant in most tissues of mice.[13] 3) Survivin 140 is the prominent variant in the damaged nerve and the only variant expressed in spinal cord. This is again in consistent with the same observation by Emadi et al., results.[19] The biological property of survivin40 variant during neurogenesis and CNS development is unclear

and need further investigation. As Survivin40 has only N-terminal domain, it may forms dimers with other variants, and modulates their function.

The expression of survivin is extensively studied by Jiang et al[17] and Emadi et al[19] during early brain development. Furthermore, the expression was also studied after traumatic brain injury (TBI) by Johnson et al.[20] Based on 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 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.[20] Our presented data along with previous findings provides a more clear picture on the involvement of survivin in normal and abnormal physiology of the nervous system. However, more works needed to elucidate the complex role of survivin in neuronal and glial cells.

Conclusion:

In conclusion, our data suggest that survivin is differentially expressed and spliced during the course of regeneration in damaged nerve and spinal cord. The data also suggest that manipulation of expression and/or splicing of survivin could potentially affect the process of regeneration in nerve and/or spinal cord injuries.

Acknowledgments:

We are grateful to Drs Mohamad Reza Soroush, Hassan Salimi and Gholamreza Salehi Jouzani for their excellent assistance and advice. This work was supported by a research grant from Iranian foundation of Janbazan Medical and Engineering Research Center (grant number 2011).

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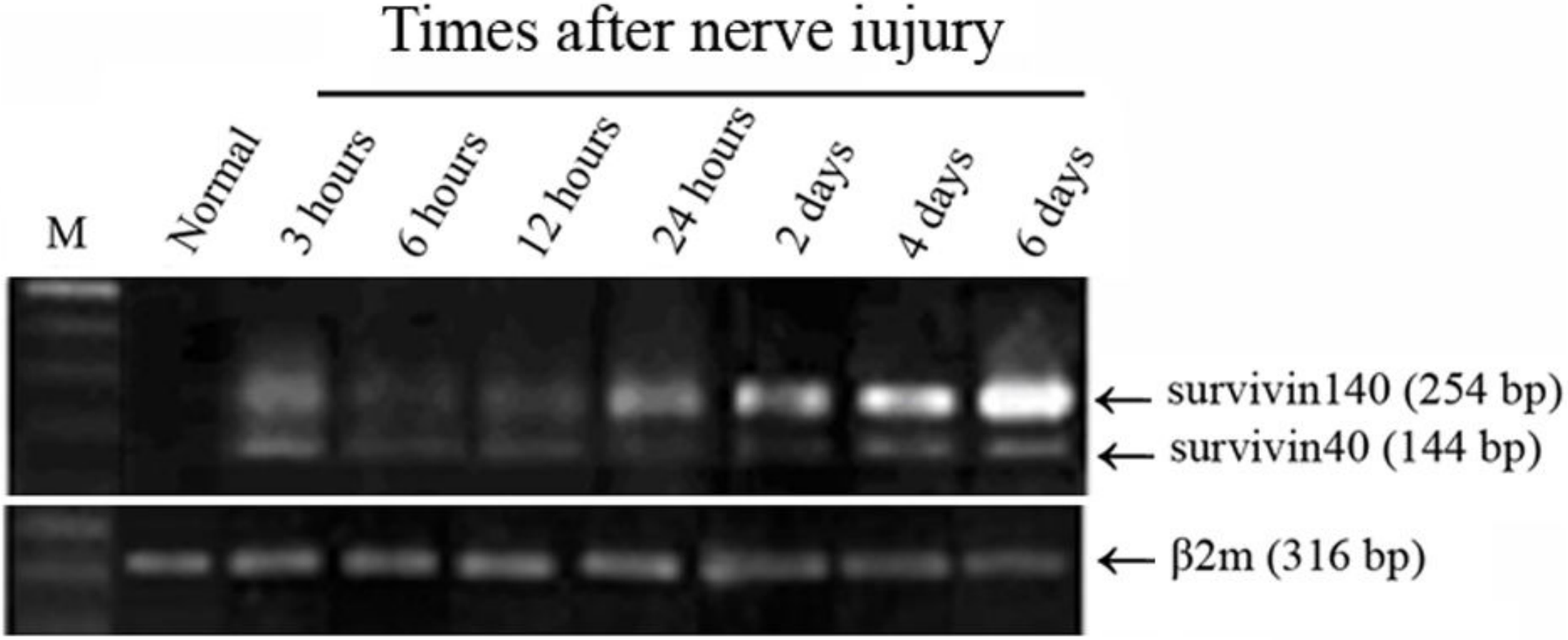
Legends:

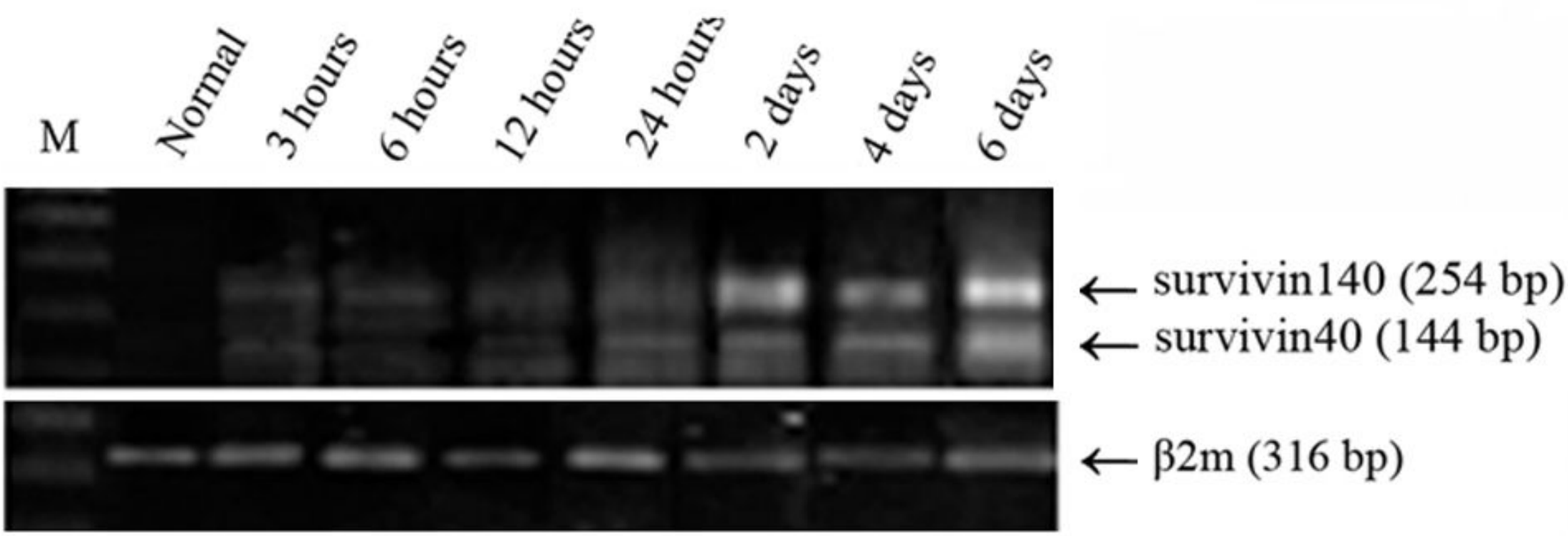
Figure 1: RT-PCR analysis of survivin gene expression in distal (A), and proximal (B) segments of dissected sciatic nerve and its corresponding (L4-L6) segments of spinal cord before and at specific time-points after injury. Note that there is no expression of survivin in undamaged (normal) nerve samples and that there is no expression of survivin40 in spinal cord samples. The lower band in each lane represents the RT-PCR products for b2m (internal control).

Figure 2: Histograms comparing the relative expression of survivin variants in normal and damaged nerve and spinal cord samples at different time points after sciatic nerve injury in: A) distal segment of dissected sciatic nerve. B) Proximal segment of dissected sciatic nerve. C: L4-L6 segments of spinal cord before and after the injury. Values are shown as the mean± S.D.

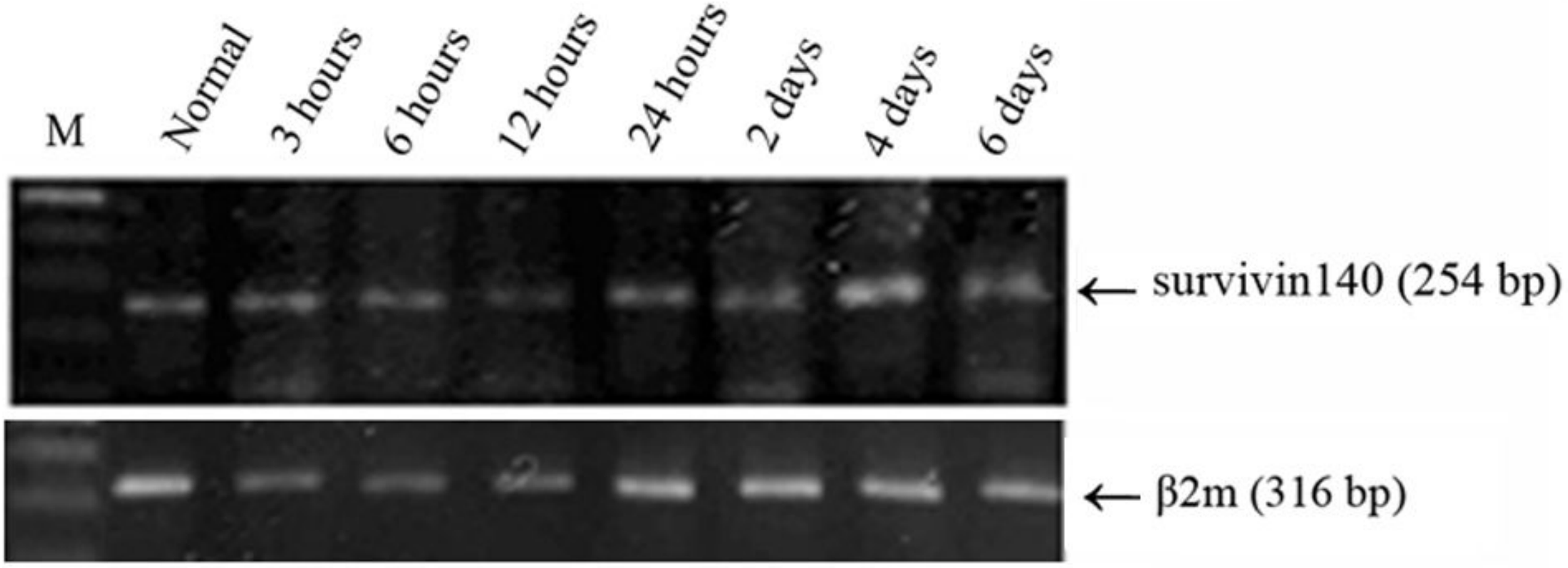
A

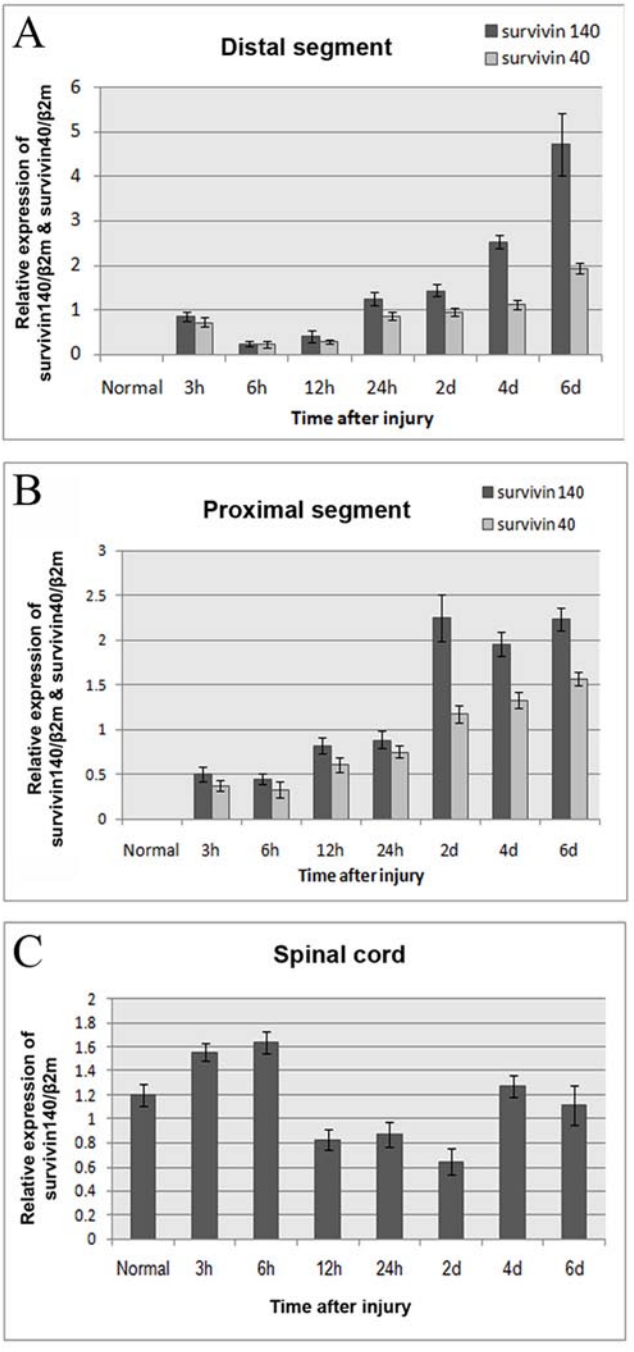

Times after nerve iujury
M
Normal
3 hours
6 hours
12 hours
24 hours
2 days
4 days
6 days
← survivin140 (254 bp)
← survivin40 (144 bp)
← β2m (316 bp)

B


M
Normal
3 hours
6 hours
12 hours
24 hours
2 days
4 days
6 days
← survivin140 (254 bp)
← survivin40 (144 bp)
← β2m (316 bp)

C


M
Normal
3 hours
6 hours
12 hours
24 hours
2 days
4 days
6 days
← survivin140 (254 bp)
← β2m (316 bp)


A
Distal segment
survivin 140
survivin 40
Relative expression of survivin140/β2m & survivin40/β2m
0
1
2
3
4
5
6
Normal
3h
6h
12h
24h
2d
4d
6d
Time after injury
B
Proximal segment
survivin 140
survivin 40
Relative expression of survivin140/β2m & survivin40/β2m
0
0.5
1
1.5
2
2.5
3
Normal
3h
6h
12h
24h
2d
4d
6d
Time after injury
C
Spinal cord
Relative expression of survivin140/β2m
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Normal
3h
6h
12h
24h
2d
4d
6d
Time after injury