J Med Biochem 2013; 32 (4) DOI: 10.2478/jomb-2013-0036

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 32: 398–405, 2013

Original paper
Originalni naučni rad

REAL-TIME PCR AND IMMUNOCYTOCHEMICAL STUDY OF CHONDROITIN SULFATE PROTEOGLYCANS AFTER SCRATCH WOUNDING IN CULTURED ASTROCYTES

PCR I IMUNOCITOHEMIJSKA STUDIJA EKSPRESIJE HONDROITIN-SULFATNIH PROTEOGLIKANA NAKON POVREDE ASTROCITA U KULTURI

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Summary

Background: Various *in vivo* and *in vitro* models have been described in order to elucidate the pathobiology underlying the traumatic brain injury (TBI) and test potentially suitable treatments. Since TBI is a complex disease, models differ in regard to the aspect of TBI that is being investigated. One of the used *in vitro* models is the scratch wound assay, first established as a reproducible, low-cost assay for the analysis of cell migration *in vitro*. The aim of the present study was to further investigate the relevancy of this model as a counterpart of *in vivo* TBI models.
Methods: We have examined the astrocytic response to a mechanical injury in terms of expression of chondroitin sulfate proteoglycans (CSPGs) – phosphacan, neurocan and brevican, using real-time PCR and immunocytochemistry.
Results: Our results indicate that *in vitro* scratch wounding alters the expression profile of examined CSPGs. Four hours after the scratch injury of the astrocytic monolayer, real-time PCR analysis revealed upregulation of mRNA levels for phosphacan (3-fold) and neurocan (2-fold), whereas brevican mRNA was downregulated (2-fold). Immunofluorescent signal for phosphacan and neurocan was more intense in astrocytes close to the injury site, while brevican was scarcely present in cultured astrocytes.
Conclusions: Obtained results indicate that CSPGs are differentially expressed by astrocytes after scratch wounding,

Kratak sadržaj

Uvod: Brojni *in vivo* i *in vitro* modeli opisani su sa ciljem da se rasvetle patobiološki procesi koji su osnova traumatske povrede mozga (TPM) i testiraju potencijalni tretmani. Imajući u vidu da je TPM kompleksno oboljenje, ovi modeli se međusobno razlikuju shodno aspektu TPM koji se ispituje. Jedan od *in vitro* modela je i povreda ćelijskog jednosloja grebanjem (engl. »scratch wound« assay), isprva ustanovljen kao ponovljiv, jeftin test za analizu ćelijske migracije *in vitro*. Cilj ove studije je da se bliže ispita relevantnost ovog modela u odnosu na *in vivo* modele TPM.
Metode: Da bi se istražio odgovor astrocita na mehaničku povredu, praćena je ekspresija odabranih hondroitin-sulfatnih proteoglikana (CSPG) – fosfakana, neurokana i brevikana, korišćenjem PCR u realnom vremenu i imunocitohemije.
Rezultati: Dobijeni rezultati su pokazali da *in vitro* povreda astrocitnog jednosloja menja profile ekspresije ispitivanih CSPG. Četiri sata nakon povrede, primena PCR u realnom vremenu analize pokazala je povećanje nivoa iRNK za fosfakan (trostruko) i neurokan (dvostruko), dok je iRNK za brevikan bila smanjena na polovinu kontrolne vrednosti. Imunofluorescentni signal poreklom od fosfakana i neurokana je bio intenzivniji u astrocitima bližim mestu povrede, dok je signal za brevikan bio slab kako u kontrolnoj, tako i u ozleđenoj grupi.
Zaključak: Dobijeni rezultati pokazuju da povreda izazvana grebanjem različito utiče na ekspresiju ispitivanih CSPG u

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demonstrating that the scratch wound model might be suitable for investigation of astrocyte-derived response to injury.

Keywords: reactive astrocytes, chondroitin sulfate proteoglycans, *in vitro* model, scratch-wound assay, gene expression

astrocitima, što ukazuju da ovaj model može biti pogodan za ispitivanje odgovora astrocita na povredu.

Ključne reči: reaktivni astrociti, hondroitin-sulfatni proteoglikani, *in vitro* model, »scratch-wound« test, ekspresija gena

Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability and a major health burden worldwide. It is generally accepted that TBI is a complex, biphasic disease consisting of primary and secondary injury. Primary injury is an immediate damage that triggers a secondary injury, which affects the brain to a greater extent, causing delayed neuronal death and neurological deficits (1). Although there is a large database of knowledge regarding processes which take place after TBI, to date no successful treatment has been defined, indicating that there is clearly a need for further studies of TBI (2, 3). In the foundations of this need, there is an urge to define appropriate models of TBI, both *in vivo* and *in vitro*.

Different *in vivo* models of TBI have been described so far and used in order to examine the background of TBI. Since the primary insult cannot be therapeutically approached, *in vivo* models are orientated toward examination and treatments of secondary damage, which is substantially recognized through a process of reactive astrogliosis. The hallmark of reactive astrogliosis is the formation of a glial scar, a barrier to axonal regeneration (4). Beside mentioned, the upregulation of several proteins, including chondroitin sulfate proteoglycans (CSPG) is reported as well (5–7). In the adult brain, basic levels of CSPGs are necessary, since they maintain the structure of extracellular matrix (ECM), stabilize synapse and proper axonal sprouting, and therefore manage plasticity (8, 9). It is believed that all of the major brain cell types contribute to the production of these normal levels of CSPG (9). However, in pathological conditions, upregulation of CSPG occurs and it is mainly attributed to astrocyte activation (10).

A number of *in vitro* models have been introduced in order to reproduce the features of interest in an isolated system and to allow manipulations, monitoring on multiple levels in real time, while being precisely controlled (11, 12). One of such is a scratch-wound assay (SW), first established as a simple, reproducible assay for the analysis of cell migration *in vitro* (13). The SW model was soon adopted by many to investigate different aspects of astroglial response to mechanical injury (14–17). As a response to SW injury, the following characteristics of reactive astrocytes have been observed: hyperplasia, enhanced expression of extracellular matrix molecules and elongation of hypertrophic processes (18, 19).

The aim of the present study was to further describe astrocyte response in an SW model, in regard to alterations in expression of CSPGs after scratch wounding *in vitro*. Therefore, using quantitative real-time PCR and immunohistochemistry, we have investigated the expression profiles of phosphacan, neurocan and brevican.

Material and Methods

The mixed astrocyte and microglia culture was prepared from Wistar pups up to 3 days postpartum obtained from the vivarium of the Institute for Biological Research »Siniša Stanković« (Belgrade, Serbia). All experimental procedures were in compliance with the EEC Directive (86/609/EEC) on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research »Siniša Stanković«, University of Belgrade.

Cell culture

The preparation of mixed astrocyte and microglia culture was done as described previously (McCarthy & De Vellis, 1980). The cultures were kept in Dulbecco's Modified Eagle's Medium, (DMEM, Gibco, InvitrogenCo, Carlsbas, CA, USA) supplemented with 10% FCS (PAA Laboratories GmbH, Austria) and 1% penicillin/streptomycin (PAA Laboratories GmbH, Austria). The medium was changed every 2–3 days. After 10–14 days, extent of microglial cells was detached from the astrocytes and remaining cells were then pelleted by centrifugation (3000 rpm for 5 min). For immunocytochemical (ICC) and morphological analysis, cells were plated at 6×10^5 on glass cover slip circles (25 mm) in 6-well plates (Sarstedt, Newton, USA) with 2 mL DMEM with 10% FCS per well. Twenty-four hours before the induction of scratch wound, medium was changed with DMEM containing 0.1% FCS. Scratch wound assay was performed as previously described (17). Briefly, confluent cell monolayers were wounded by scratching with a sterile pipette (yellow) tip, inducing three (for qRT-PCR analysis) or two (for ICC analysis) parallel scratches per well. The number of microglial cells and astrocytes in each culture was evaluated by double immunofluorescence staining against IBA1 (Serotec, mouse, 1:400) and GFAP (DAKOCytomation, rabbit, 1:700).

Real-time PCR analysis

For RNA extracts, 8x106 cells were plated in six-well plates (Sarstedt, Newton, USA). Total RNA was isolated using the PeqGold RNA Pure reagent (Peqlab, Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's recommended protocol. RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCR (qRT-PCR) was conducted using SYBR Green technology (Applied Biosystems, Carlsbad, CA, USA) and analyzed on an AbiPrism 7000 (Applied Biosystems, Carlsbad, CA, USA). Primer pairs for phosphacan, neurocan, brevican and cyclophilin-A were from Invitrogen, Germany (*Table I*). The target gene expression levels were determined by the comparative 2^ (-delta Ct) quantification method using cyclophilin-A as a reference gene.

Phase-contrast microscopy and immunocytochemical (ICC) analysis

Phase-contrast and ICC analyses were performed using standard protocol. Live cells were used for phase-contrast imaging, at different time points after scratch injury. Briefly, for ICC analyses, cells were fixed for 20 min in 4% paraformaldehyde (PFA) at 4 °C. Permeabilization was performed in 0.25% Triton X – 100 for 10 min, followed by blocking of nonspecific staining in 1% BSA for 30 min. Double labeling procedure was performed with the following primary antibodies: anti-IBA1 (Abcam, Cambridge, MA, USA, goat, 1:500), anti-phosphacan (Chemicon, mouse, 1:400), anti-neurocan (Santa Cruz, mouse, 1:400), and anti-brevican (Santa Cruz Biotechnology, Santa Cruz, CA, USA, goat, 1:400) with anti-GFAP (DAKO Cytomation, Denmark, rabbit, 1:700), overnight at 4 °C. Visualization of reaction was obtained using appropriate secondary fluorescent antibodies (1:250, Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 donkey anti-goat, and Alexa Fluor 555 donkey anti-rabbit, Invitrogen, Grand Island, NY, USA) and nuclei were marked using DAPI (Invitrogen, Grand Island, NY, USA). The cells were cover-slipped with Mowiol (Calbiochem, San Diego, CA, USA). To test the specificity of the reaction, cell cultures were treated in the same way with the omission of the primary antibodies. Phase-contrast imaging and immunofluorescence were examined and photographed with Carl Zeiss Axiovert microscope (Zeiss, Gottingen, Germany). Cell count for astrocyte/microglia ratio was performed in seven quadrants (0.38 mm^2) per each specimen from three independent experiments (n=21).

Table I List of primers used for real-time polymerase chain reaction.

Primer	Sequence
Cyclophilin-A	Forward – GGCAAATGCTGGACCAAACAC Reverse – TTAGAGTTGTCCACAGTCGGAGATG
GFAP	Forward – CTCCTATGCCTCCTCCGAGACGAT Reverse – GCTCGCTGGCCCGAGTCTCTT
Brevican	Forward – CCTCAGGAAGCTGTGGAGAG Reverse – CTTGCCCCATCTGGAGTAGA
Phosphacan	Forward – TTGACAAGTGATGAAGAGAGTGG Reverse – AATCAGCACATCTCGTTCTATCC
Neurocan	Forward – TTTCAGTCCACAGCGATCAG Reverse – AGGAGAGGGATACAGCAGCA

Statistical analysis

Data obtained with qRT-PCR analysis are shown as means ± SEM. Statistical significance of differences between the groups was determined using the one-way analysis of variance (ANOVA for repeated measures) whereas P values less than 0.05 ($p<0.05$) were considered statistically significant.

Results

General characteristics of the SW model and mixed glial cell culture

Astrocytic monolayer was examined up to 48 hours after the SW (for schematic representation, see *Figure 1A*), using phase-contrast microscopy (*Figure 1B*). These results showed the characteristic morphological features and migration trend of astrocytes at the injury site in the given time points (4, 24 and 48 hours after the SW). In order to determine the ratio of astrocyte/microglia in mixed glial culture, staining with IBA1 and GFAP was performed (*Figure 1C*). Results indicated that this ratio is approximately 9:1 in favor of astrocytes (*Figure 1D*).

Real-time PCR analysis of CSPGs expression profiles in cultured astrocytes before and after SW

Gene expression analysis showed that all three proteoglycans analyzed herein are expressed in mixed astrocyte-microglia culture (*Figure 2*). However, the results showed that there is a distinct difference in abundance of neurocan, phosphacan and brevican mRNAs when expressed relative to cyclophilin-A. Neurocan-mRNA was 2 times more abundant than phosphacan-mRNA and 14 times more abundant than brevican-mRNA. The qRT-PCR results also demonstrated that GFAP mRNA content remained unchanged after the SW. Four hours following the SW, both phosphacan and neurocan mRNAs were significantly upregulated (2.73±0.66, 5.2±1.2, respectively) when compared to the control group (0.94± 0.27, 2.2±0.76, respectively, $P<0.05$). In contrast, the level of brevican mRNA dropped considerably (0.08±0.007) below the control values (0.17±0.11, $P<0.05$).

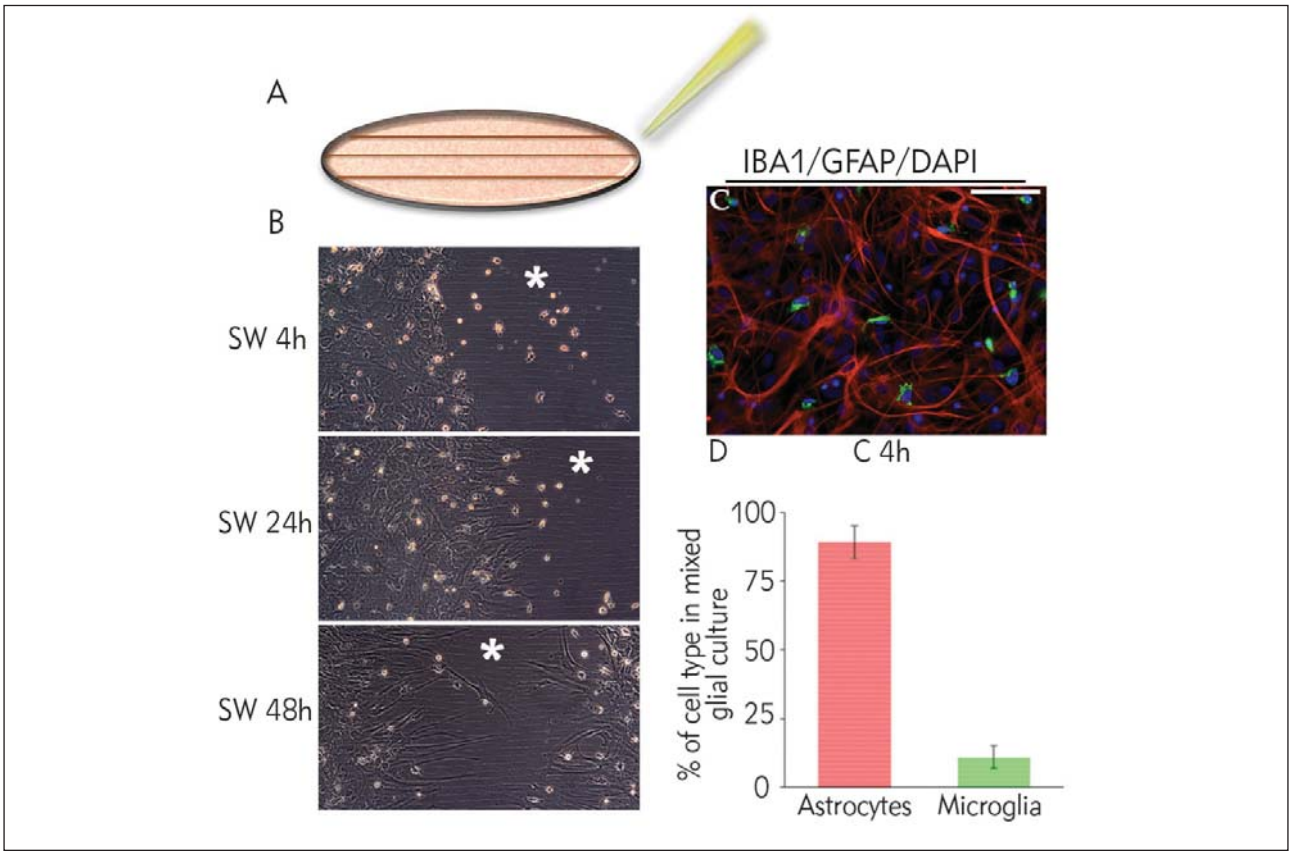

Figure 1 General characteristics of SW model and mixed glial cell culture.

(A) Schematic preview of SW injury. (B) Phase-contrast imaging at 4, 24 and 48 hours after the injury revealed the expected astrocytic response to scratch. Injury site is marked with an asterisk. (C) Participation of astrocytes and microglia in mixed glial cell culture is determined by double immunofluorescence staining for GFAP (red) and IBA1 (green), respectively. Nuclei are stained with DAPI (blue). Scale bar: 30 μm. (D) Astrocytes/microglia ratio was approximately 9:1 (n=21).

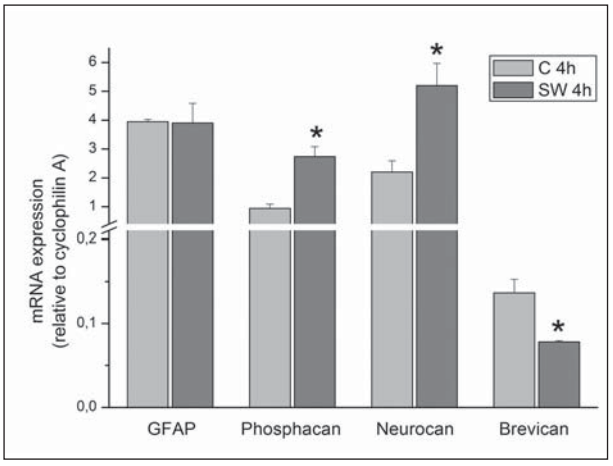

Figure 2 The effect of SW on GFAP, phosphacan, neurocan and brevican mRNA expression determined by qRT-PCR. Levels of each CSPGs mRNA were expressed relative to the expression of cyclophilin-A used as a housekeeping gene. Proteoglycans mRNA relative expression values four hours after the scratch wound (SW 4h) were compared to corresponding control groups (C 4h) and presented as mean ± SEM. The values of *P<0.05 were considered statistically significant.

Effect of SW on neurocan, phosphacan and brevican immunosignal in cultured astrocytes

To further depict the expression of neurocan, phosphacan and brevican after the SW at a given time point, double immunofluorescence analysis together with DAPI fluorescence counter-labeling was performed (*Figure 3*, injury site is marked with a dashed line). In control samples, phosphacan and neurocan were occasionally detected in the cytosol of GFAP positive astrocytes (*Figure 3A-D, I-L*). Consistent with low mRNA expression, brevican was scarcely present in some astrocytes in control samples (*Figure 3Q-T*). Immunofluorescent signal for phosphacan and neurocan was enhanced four hours after the SW, and intensive labeling was seen in the majority of astrocytes surrounding the injury site (*Figure 3E-H, M-P*). At the same time point, no difference in immunofluorescent intensity was seen for brevican (*Figure 3U-X*).

Discussion

Although *in vitro* models of TBI cannot be considered a substitute for *in vivo* models, they are

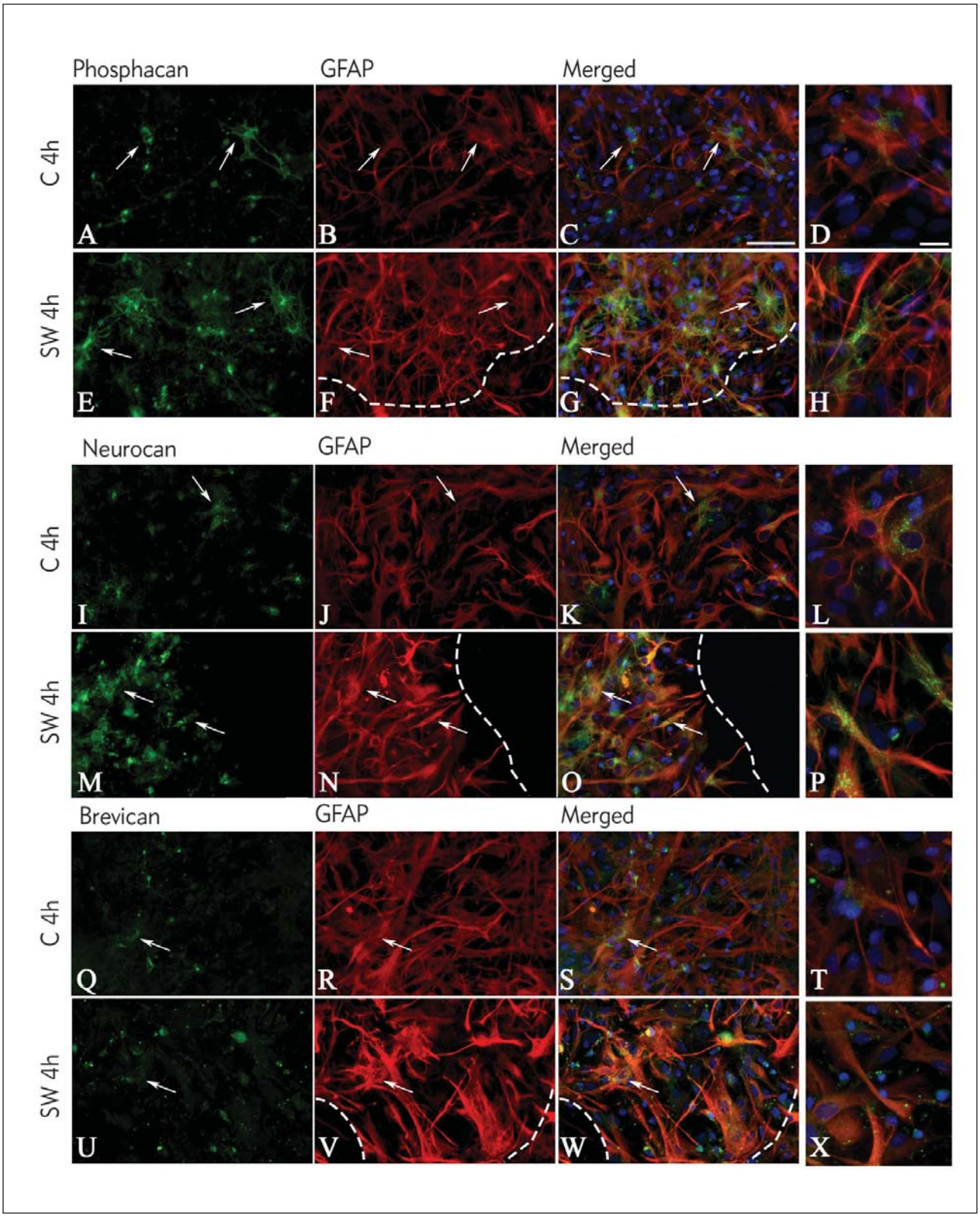

Figure 3 Double immunofluorescence staining of phosphacan, neurocan and brevican (green) and GFAP (red). Nuclei in overlapped images are marked with DAPI (blue). In control groups phosphacan and neurocan immunofluorescence were detected in cytosol of some astrocytes (A-D, I-L, arrows), while brevican was scarcely present (Q-T, arrows). At SW 4h, most of the astrocytes close to the injury site were stained positive for phosphacan and neurocan (E-H, M-P, arrows). Compared to control, brevican immunofluorescence staining was slightly changed at SW 4h (U-X, arrows). SW site is marked with a dashed line. Scale bars: 30 μm in lower magnification and 10 μm in higher magnification.

advantageous complements when it comes to exploration of isolated phenomena. Moreover, *in vitro* models are easy to use, affordable, less ethically challenged, reproducible and better controlled, and represent a good platform for drug screening (21–23). Hence, *in vitro* models of TBI are a variable tool in studying the pathobiology beyond brain injury and there is a need to define them more profoundly.

Reactive astrogliosis is an event that occurs *in vivo* in response to all forms of brain injury. Typical biochemical hallmark of reactive astrogliosis is increased expression of intermediate filament GFAP (4, 12). However, it is important to stress that *in vitro* injuries to astrocytes somewhat vary in this sense and give rise to opposite results. For instance, results from experiments with chemical treatments of astrocytes (dibutyryl cyclic AMP, interleukin-1β, or macrophage-conditioned medium) suggest insignificant changes in GFAP (12). However, the elevation of GFAP content by about 70% was estimated in a »scraping« model of injury to astrocytes (18). It was also reported that, twenty-four hours after the SW, some of the astrocytes close to the wounding site displayed elevated GFAP-immunoreactivity (17). On the contrary, Yang et al. (15) reported no change in GFAP content analyzed by immunoblot up to 5 days after the SW. Our results indicate that mRNA levels for GFAP remain unchanged 4 hours after the SW injury to astrocytes. However, ICC analysis of GFAP expression revealed an enhanced immunopositive signal for GFAP in astrocytes surrounding the scratch site. Moreover, astrocytes close to the injury site gained characteristic morphological features of reactive astrocytes: hypertrophia and elongation of processes. Similar results were previously obtained after a mechanical stretch of differentiated astrocytes with addition of meningeal fibroblasts, where increases in peak of GFAP immunofluorescence did not match increases in *de novo* GFAP expression (24). Wanner et al. (24) contemplated that GFAP accumulation in astrocyte processes may partially be a result of cell contraction and cytoskeletal fasciculation. Taken together, these results suggest that GFAP is unreliable as a marker for astrocytes activation *in vitro*. It is also known from *in vivo* models that TBI causes accumulation of CSPGs which contribute to the formation of a barrier that prevents axonal regeneration through the injured site (7, 25, 26). Moreover, it is thought that reactive astrocytes are the primary source of these important inhibitory proteins (10). Previous studies suggest that all members of the CSPG family are upregulated after CNS injury (27, 28), although this alteration varies in terms of examined structure and time point after the injury (29). Since phosphacan, neurocan and brevican have been recognized as markers of post-injury scar formation, the main focus of this study was to estimate the dynamics of their expression in astrocytes, four hours after the SW injury. Our qRT-PCR data showed that scratching of mixed astrocyte-microglial culture led to increased expression of neurocan and phosphacan, whereas transcript levels of brevican dropped below control values. Furthermore, ICC analysis revealed that all three CSPGs where expressed in astrocytes and not in microglia.

The most abundant mRNA transcript of herein examined CSPGs was for neurocan. Our results indicate that cultured astrocytes produce neurocan and that the SW injury induced substantial upregulation of neurocan mRNA, which is consistent with previous *in vitro* (30) and *in vivo* (6, 29, 31) observations.

Another major component of the CSPG family, phosphacan, is highly upregulated following different types of CNS injury *in vivo* (32–34). Although some studies indicate that phosphacan is downregulated immediately after the injury (29), we have found upregulation 4 hours after the SW which was consistent with previously published results of *in vitro* injury in astrocytes (24). Our ICC data revealed that both neurocan and phosphacan immunoreactivity was seen in the perinuclear area of control astrocytes. SW induced enhanced staining in the elongated processes of astrocytes surrounding the scratch site, similar to previous descriptions (24).

Although it has been confirmed both *in vivo* and *in vitro* that astrocytes are the major source of brevican (35–37), results of qRT-PCR analysis from our cultures suggested that brevican transcript was drastically less present compared to neurocan and phosphacan. It is important to note that SW induced a decrease in expression to about half of the value seen in control. Similar drop in brevican mRNA and protein levels below control was reported in different *in vivo* models of brain (37, 38) and spinal cord injuries (39).

In conclusion, we have demonstrated that after 4 hours, the SW injury in mixed astrocyte-microglial culture induced 1) astrocyte stellation and migration to the injury site, 2) upregulation of phosphacan and neurocan and downregulation of brevican in astrocytes, and 3) enhanced staining of GFAP in astrocytes close to the scratch site without affecting GFAP mRNA levels.

Therefore, the present system may be considered as a good model of an *in vitro* glial scarring and may be useful for elucidating mechanisms beyond its formation as well as for testing potential therapeutic approaches.

Acknowledgment. This study was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, grant number III41014.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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Received: June 14, 2013

Accepted: July 15, 2013