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## Upregulation of heparin-binding growth-associated molecule after spinal cord injury in adult rats<sup>1</sup>

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**KEY WORDS** heparin-binding growth-associated molecule; spinal cord injuries; immunohistochemistry; reverse transcriptase polymerase chain reaction; astrocytes; neurons

### ABSTRACT

**AIM:** To investigate whether traumatic spinal cord injury (SCI) induces changes of gene expression of heparin-binding growth-associated molecule (HB-GAM). **METHODS:** In a spinal cord transection model, HB-GAM expression and cellular localization were examined using Northern blot, RT-PCR, immunohistochemistry and immunofluorescence double-labeling methods. **RESULTS:** HB-GAM mRNA was significantly upregulated in spinal cord tissues rostral and caudal to the injury at 7 d after SCI. HB-GAM gene expression was markedly increased at 3 d, peaked at 7 d, and declined to the baseline level at 28 d post-injury. During its peak expression, HB-GAM was co-localized in astrocytes, oligodendrocytes, and neurons in spinal cord tissues within 7 mm from the site of spinal transection. **CONCLUSION:** SCI induces HB-GAM expression at both mRNA and protein levels in areas close to the injury. Both neurons and glial cells expressed HB-GAM implying that HB-GAM played a role in the process of injury and/or repair following SCI.

### INTRODUCTION

The neurite-growth promoting protein heparin-binding growth-associated molecule (HB-GAM)<sup>[1]</sup> was purified from rat brain and has been shown to express during early postnatal brain development when glial and neuronal cells are actively differentiating *in vivo*. HB-GAM is an 18-kDa molecule and has been suggested to play an important role in promoting neurite outgrowth

in the developing brain<sup>[2]</sup>. Cloning of the corresponding mitogenic protein pleiotrophin<sup>[3]</sup> (PTN) revealed the same sequence, indicating that the proteins designated as PTN and HB-GAM are identical.

In addition to enhancing neurite outgrowth in a developmentally regulated manner, HB-GAM is accumulated at the growth cone-target interface accompanying the onset of synaptogenesis, as evidenced by its presence at the neuromuscular junction of the *Xenopus* and rat<sup>[4]</sup>. *In vitro* studies have suggested that HB-GAM functions as a local, synaptic matrix-associated factor that enhances both presynaptic and postsynaptic differentiation during development<sup>[5]</sup>. HB-GAM expression is significantly increased in neurons of the hippocampus, piriform cortex, and the parietal cortex after chemically induced seizures<sup>[6]</sup>, indicating its po-

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tential for activation of cells in the central nervous system (CNS). High-frequency stimulation leading to induction of long-time potentiation resulted in increased expression of HB-GAM in the CA1 region of the rat hippocampus, indicating that HB-GAM plays a role in the modulation of synaptic function in the hippocampus<sup>[7]</sup>. HB-GAM expression in reactive astrocytes can be induced following hippocampal neuronal injury<sup>[8]</sup> suggesting that HB-GAM, produced by the reactive astrocytes, may participate in the repair process after CNS injury.

Traumatic spinal cord injury (SCI) has a devastating impact on patients resulting from the complete or partial loss of motor and sensory function below the level of lesion. One important aspect of SCI research is to screen therapeutic molecules that may exert neuroprotective or neuroregenerative effects. Due to the facts that HB-GAM expresses during development, promotes neurite outgrowth, and enhances synaptogenesis, we reasoned that this molecule might have re-expressed following SCI and played a reparative role. In the present study, we investigated whether HB-GAM expressed following SCI and, if so, what cell types contributed to its expression. Our long-term goal is to investigate whether HB-GAM contributes to axonal regeneration and synaptic reconnection following SCI.

## MATERIALS AND METHODS

**Spinal cord transection** Adult Sprague-Dawley female rats weighing 220-250 g were anesthetized by ip injection of sodium pentobarbital (40 mg/kg, Anthony Products Co, Arcadia, CA). After a laminectomy was performed at the 9th thoracic (T9) level ( $n=20$ ), the underneath spinal cord was exposed and transected completely. After the transection, the muscles and skin were closed in layers, and the rats were placed in a temperature- and humidity-controlled chamber. Manual bladder emptying was performed at least three times daily until reflex bladder emptying was established. In the control group ( $n=20$ ), rats received laminectomy only with no spinal cord transection. All animal handling, surgical procedures, and post-operative care were approved by the Animal Use and Care Committee of the Shanghai Second Medical University and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA).

**Perfusion and tissue preparation** Rats were

sacrificed at 1, 3, 7, 14, and 28 d ( $n=3$  per time point) post-injury by an overdose of sodium pentobarbital (60 mg/kg) followed by an intracardiac perfusion of 4 % paraformaldehyde in cold HBSS. After perfusion, a 14-mm long spinal segment containing the injury epicenter was removed carefully, post-fixed in the same fixatives overnight at 4 °C, and cryoprotected with 30 % buffered sucrose. Horizontal sections of the spinal cord containing the injury epicenter were cut at 14  $\mu$ m on a cryostat and were mounted on poly-*L*-lysine-coated slides and stored frozen prior to immunohistochemistry.

**Northern blot analysis** Tissues obtained from the injured ( $n=5$ ) and sham-operated spinal cords ( $n=5$ ) were used for total RNA extraction. Total RNAs were prepared using TRIzol reagents (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Thirty microgram of the total RNA from each tissue sample was loaded on each gel lane, separated by electrophoresis, and transferred onto a nylon membrane. The HB-GAM probe and an internal control probe encoding GAPDH were labeled at the 3' end by [ $\alpha$ -<sup>32</sup>P] dATP (NEN, Boston, MA) using terminal deoxynucleotidyl transferase (Amersham Pharmacia, Piscataway, NJ) and purified. Prehybridizations were performed for 30 min at 68 °C in ExpressHyb Solution (Clontech, Palo Alto, CA). The column-purified and denatured probes were then added and hybridized at 68 °C for 1 h. After hybridization, the membrane was washed in 2 $\times$ SSC/SDS 0.5 g/L for 40 min for several times at room temperature, and then washed twice in 0.1 $\times$ SSC/SDS 1 g/L at 50 °C for 40 min. Then the membrane was exposed to Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) for 24 h and the image was acquired.

**RT-PCR** Total RNA was extracted using Trizol solution according to the manufacturer's protocol. Possible DNA contamination was removed by DNase treatment. Two microgram total RNA was used to prepare cDNA using Superscript II RNase H reverse transcriptase (Life Technologies, Gaithersburg, MD). The house-keeping gene GAPDH was used as internal control. After digested with RNase H (1  $\mu$ L) for 20 min at 37 °C, the cDNAs were then amplified by PCR. The synthetic oligonucleotides were obtained from Shanghai Sangon Co Ltd. PCR was performed to amplify 210 bp product for HB-GAM and 579 bp product for GAPDH. Primers: HB-GAM, 5'-AGAATGGCAATGGAGTGT-3', 5'-CTGGTCTTCAAGGCGGTA-3'; GAPDH, 5'-TCGGTGTGAACGGATTTG-3', 5'-TCTTCTGAGTGGCA-

GTGAT-3'. PCR reaction 50  $\mu$ L contained KCl 50  $\mu$ mol/L, Tris-HCl 10 mmol/L (pH 8.3), MgCl<sub>2</sub> 1.5 mmol/L, each dNTP 0.2 mmol/L, each primers 0.6 mmol/L for HB-GAM, each primers 0.06 mmol/L for GAPDH and 2 U Hotstar *Taq*<sup>TM</sup> DNA polymerase (Qiagen, Valencia, CA). Reaction mixture was incubated for 10 min at 95 °C, and then subjected to 34 cycles of melting (45 s at 94 °C), annealing (30 s at 60 °C), and elongation (45 s at 72 °C). The final extension was for 10 min at 72 °C. Then aliquots were run on 12 g/L agarose gels containing ethidium bromide, then photographed under UV illumination.

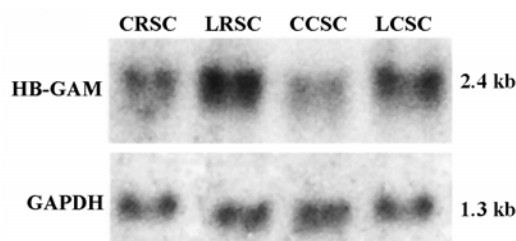
**Immunohistochemistry** HB-GAM immunohistochemistry was performed using goat anti-HB-GAM (Santa Cruz Biotechnology, Santa Cruz, CA). ABC system was used with 3,3'-diaminobenzidine hydrochloride (DAB) as the chromagen to detect the presence of HB-GAM protein. Endogenous peroxidase activity was blocked by treatment with 0.75 % H<sub>2</sub>O<sub>2</sub> in PBS for 10 min. Slides were placed in Triton X-100 1 g/L for 30 min, washed in PB 0.1 mol/L, exposed to normal goat serum 100 g/L for 30 min, then anti-HB-GAM IgG at 1:100 overnight at 4 °C. After washing, the slides were then incubated in biotinylated rabbit anti-goat IgG (Vector, Burlingame, CA. dilution: 1:200) for 45 min, washed, incubated in streptavidin-horseradish peroxidase (Vector, Burlingame, CA) for 30 min, washed again, then developed with 0.5 g/L DAB and 0.3 % H<sub>2</sub>O<sub>2</sub> for 5 min. The slides were then washed, cleared through ascending alcohols and xylene, mounted in Permount, and photographed using an Olympus BX-60 photomicroscope. Control slides were treated similarly except for the substitution of normal goat serum for the primary antibody; no cellular labeling was observed in these sections.

**Immunofluorescence double-labeling** Immunofluorescence double-labeling was used for cellular colocalization of HB-GAM. After the sections were incubated overnight at 4 °C in anti-HB-GAM IgG at 1:100, they were washed in PBS, and incubated further with FITC-conjugated rabbit anti-goat IgG for 1 h in 37 °C. These sections were then washed three times with PBS and incubated with specific cell marker antibodies overnight at 4 °C. The cell-specific monoclonal antibodies included mouse anti-gial fibrillary acidic protein antibody (GFAP; 1:200, Sigma) to identify astrocytes, mouse anti-RIP antibody (1:50, a gift from Dr Scott R WHITTEMORE, University of Louisville) to identify oligodendrocytes, and mouse anti-OX42 an-

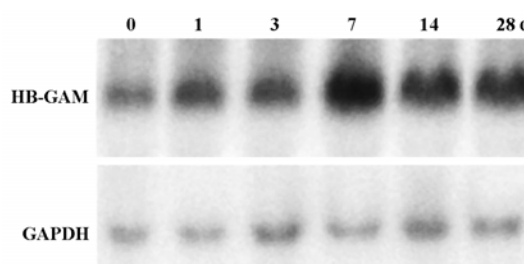
tibody (1:50; Harlan Sera-lab, Sussex, England) to recognize microglial cells, anti- $\beta$ III-tubulin (1:400; Sigma) to identify neurons. The sections were then washed with PBS and incubated further with rhodamine-conjugated secondary antibodies for 1 h at 37 °C. The sections were coverslipped with Gelmount (Sigma) for observation using an Olympus BX-60 microscope.

## RESULTS

**Upregulation of HB-GAM gene expression after SCI** GAPDH mRNA in the lesioned tissue did not change as compared to the normal controls. HB-GAM gene expression in lesioned rostral spinal cord (LRSC) and lesioned caudal spinal cord (LCSC) was up-regulated. Upregulation of HB-GAM in LRSC was higher than that in LCSC (Fig 1). In contrast, the expression of HB-GAM gene was markedly upregulated at 3 d, peaked at 7 d, and then decreased and returned to the baseline level at 28 d post-injury (Fig 2). RT-PCR was performed to further confirm the results of Northern blot. RT-PCR of GAPDH gene was carried out to test the RNA integrity and the efficiency of reverse-transcriptase reaction of each sample. HB-GAM gene expression from the injured spinal cord tissue was mark-

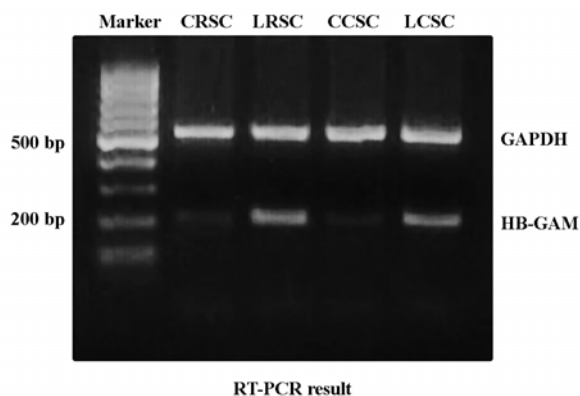


**Fig 1.** Northern blot result showing the upregulation of HB-GAM gene expression (7 d after injury). CRSC: control rostral spinal cord; LRSC: lesioned rostral spinal cord; CCSC: control caudal spinal cord; LCSC: lesioned caudal spinal cord.



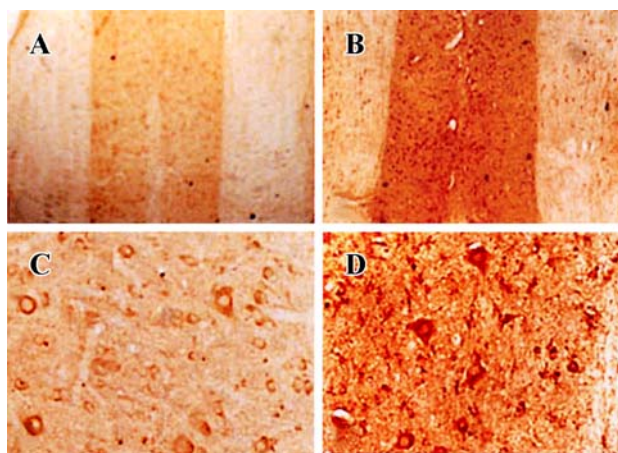
**Fig 2.** Time course of HB-GAM gene expression after spinal cord injury.

edly elevated after the injury whereas the GAPDH gene expression remained unchanged, confirming the Northern blot results (Fig 3).



**Fig 3.** RT-PCR analysis of the HB-GAM gene expression (7 d after injury). CRSC: control rostral spinal cord; LRSC: lesioned rostral spinal cord; CCSC: control caudal spinal cord; LCSC: lesioned caudal spinal cord.

**Cellular localization of HB-GAM protein** In control spinal cord sections, HB-GAM protein expression was found predominately in neurons with lower or no expression in glial cells. At 7 d post-SCI, HB-GAM immunoreactivity was markedly increased in both neurons and glial cells adjacent to the injury (Fig 4). The labeling was the strongest at and near the site of injury and was gradually decreased with increases in distance away from the injury.



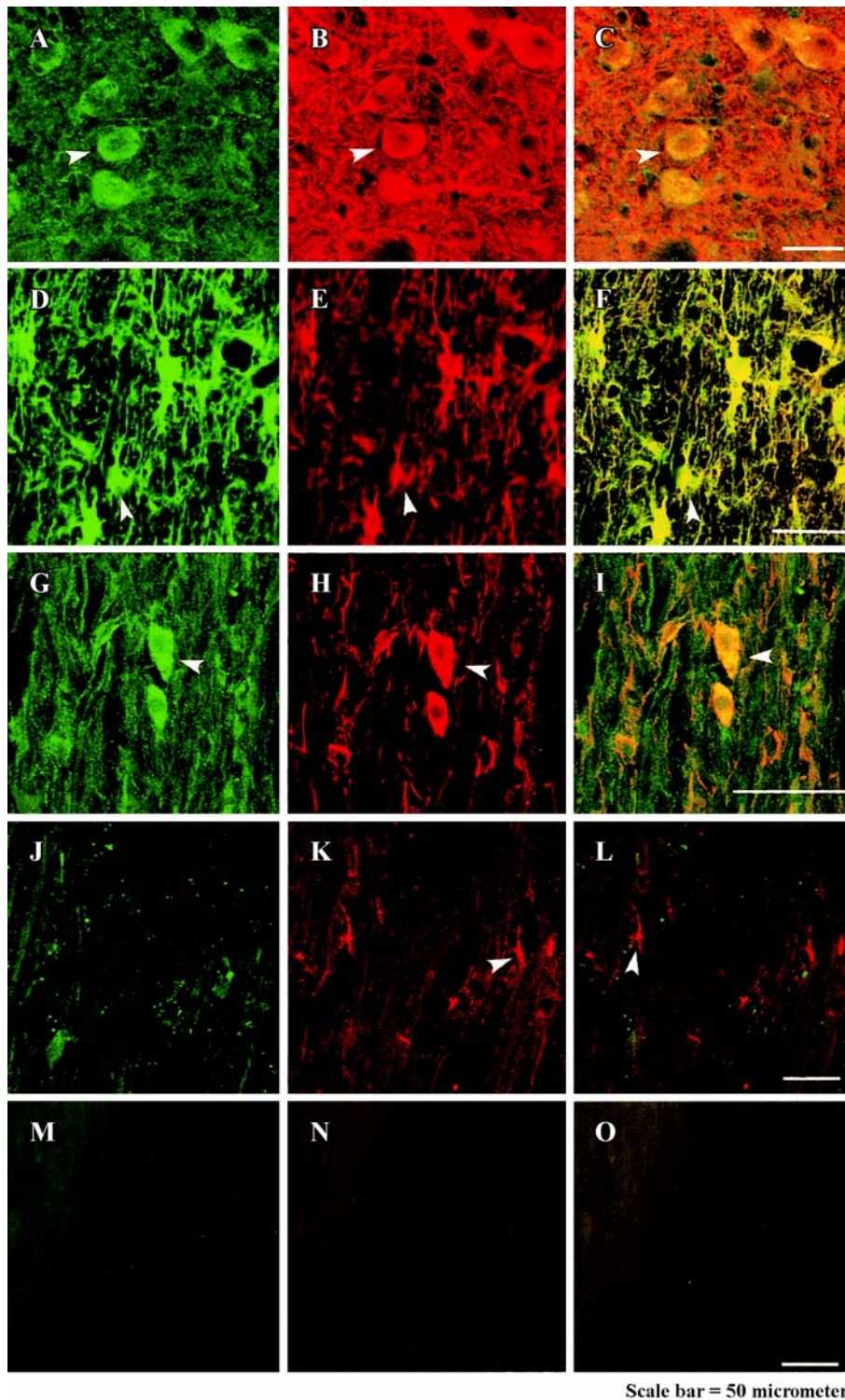
**Fig 4.** HB-GAM-immunoreactivity in the spinal cord before and after SCI (7 d after injury). (A, C) show the staining of normal spinal cord; (B, D) show the staining of injured spinal cord 7 d after SCI. Magnification: (A,B)  $\times 10$ ; (C,D)  $\times 50$ .

Immunofluorescence double-labeling was performed to identify specific cell types that express HB-GAM. In control spinal cords, HB-GAM-labeled signal was very weak, indicating low level expression of HB-GAM in control cord tissues (data are not shown). In contrast, HB-GAM protein expression was greatly increased at 7 d post-injury and was co-localized to  $\beta$ III-tubulin-positive neurons, GFAP-positive astrocytes, and Rip-positive oligodendrocytes (Fig 5). The astrocytes and oligodendrocytes were hypertrophic in appearance and exhibited strong GFAP (+) and Rip (+) staining respectively. Interestingly, HB-GAM was not co-localized to OX-42-positive microglial cells (Fig 5).

## DISCUSSION

In the rat brain, expression of HB-GAM protein is relatively low in the embryonic phase, it increases to maximal levels during the early postnatal growth phase, which is characterized by extensive outgrowth of axons and dendrite, and then dramatically decreases with maturation<sup>[1,9]</sup>. During the perinatal stage, HB-GAM is seen in cells of neural as well as glial origins. In the adult brain, HB-GAM expression is restricted to selective neuronal subpopulations, including cerebral cortex and hippocampal CA1 pyramidal neurons. In the spinal cord, the HB-GAM protein is predominately expressed in neurons but not in glial cells and other cells. It is interesting to note that, after injury, expression of HB-GAM in glial cells became evident again and the expression preferentially distributed in regions close to the injured site. The identification of HB-GAM expression in astrocytes and oligodendrocytes following SCI may indicate that the injured spinal cord reverts to a developmental pattern of glial HB-GAM expression. Reactive gliosis, which is characterized by the proliferation of reactive astrocytes and activated microglia, is a common response to the central nervous system injury. Many of the neuropeptides, transmitters and growth factors have been detected in reactive astrocytes following brain injury<sup>[10]</sup>. In the present study, HB-GAM has also been detected in reactive astrocytes and oligodendrocytes after SCI. It has been suggested that reactive gliosis may be involved in a self-repairing process at early post-injury stages.

In the present study, we demonstrated that HB-GAM expression increased greatly after spinal cord total transection. The data presented here imply that the expression of HB-GAM in neurons following SCI might



Scale bar = 50 micrometer

**Fig 5.** Confocal laser microscopy after double staining with HB-GAM and cell marker antibodies in the spinal cord at 7 d after SCI. White arrowheads indicate cells double stained for HB-GAM (green) and respective cell marker (red),  $\beta$ III-tubulin (A-C), GFAP (D-F), Rip (G-I), and OX-42 (J-L). (C, F, I, L) show the merge pictures (yellow). (M-O) show the control slides without primary antibodies.

be an intrinsic reparative mechanism aiming at promoting injured axons to regenerate. The expression of HB-GAM in glial cells including reactive astrocytes and oli-

godendrocytes suggests that the HB-GAM may also play a role in modifying glial environment involving secondary injury and/or repair processes. Whether HB-GAM

plays a role in axonal regeneration and synaptogenesis, as it does during development, needs to be elucidated in future studies.

It should be noted that HB-GAM expression in surviving neurons around the injury center was also upregulated. Surviving neurons do not extend axons by default but need to be specifically signaled by growth factors to extend processes<sup>[11]</sup> after injury. HB-GAM might have a fundamental effect on the outgrowth of processes. However, the effect may not be strong enough to induce considerable regeneration. It was demonstrated that transplants of fibroblasts genetically modified to express brain-derived neurotrophic factor promoted regeneration of adult rat rubrospinal axons<sup>[12]</sup>. Further studies will focus on modifying grafted cells, such as Schwann cells and oligodendrocyte progenitor cells, to overexpress HB-GAM to test their abilities in promoting axonal growth and synaptogenesis after SCI.

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