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Chapter 15

PROTECTIVE EFFECT OF BMP-7 OVEREXPRESSION VIA ADENOVIRUS-MEDIATED TRANSFER ON SPINAL CORD INJURY IN CULTURE AND *IN VIVO*

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ABSTRACT

Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, are important in nerve development and response to injury. Several lines of evidence suggest that BMPs selectively enhance neuronal connection in neuronal cultures and appear to improve motor function in focal ischemia and in sciatic nerve injury. This study examines whether BMP7 overexpression by adenoviral (Ad) gene transfer is able to protect the spinal cord from injury in cultures and *in vivo*. Infection of Ad-BMP7 to spinal cord cultures significantly enhanced neuronal survival, consistent with the published effects of BMPs. Ad-BMP7 transduction effectively reduced cell damage caused by menadione, a strong oxidizing agent. Furthermore, BMP7 overexpression effectively reduced lipopolysaccharide stimulation in both neuronal and glial cultures. We further utilized the NYU weight-drop device to induce spinal cord contusion (SCI) in rats. Immediately after dropping the impactor from a height of 25 mm onto T9-T10 spinal segments, Ad-BMP or Ad-GFP, as a mock control, was infused into the cords. Transgene expression was found in both Ad-transduced spinal cord and remote neuronal cell bodies in the midbrain and motor cortex, indicating Ad transduction by a retrograde transport. Nerve fibers were more preserved in the Ad-BMP7-transduced cord than in Ad-GFP-transduced cord. Interestingly, enhanced BMP7 expression significantly improved hindlimb functional recovery in Ad-BMP7-transduced SCI rats. Taken together, BMP7 secreted by Ad-BMP7 transduced cells was neuroprotective and beneficial to functional recovery after contusive spinal cord injury in rats.

Keywords: Recombinant adenovirus, BMP7, neuroprotection, gene therapy, spinal cord injury

INTRODUCTION

Traumatic spinal cord injury (SCI) is devastating and frequently causes permanent neuronal deficits. SCI disrupts long-projection axons and initiates a series of primary and secondary injury cascades. Little spontaneous regeneration occurs after SCI and current therapies for SCI are limited. Biomedical research has been directed at minimizing the impact of secondary injury and promoting neural regeneration. Following SCI, the exogenous application of neurotrophic factors at the injured site has been extensively studied and found to promote axonal regeneration and functional restoration (Mason, 2007, Cuevas et al., 1995, Huang et al., 2011). However, there have been major problems in delivering neurotrophic factors into the nervous system. Furthermore, it is not known whether growth factors can modulate neuronal responses to spinal cord injury over the extended distance.

Bone morphogenetic proteins (BMPs), members of the TGF-beta subfamily of growth factors, have been implicated in nervous system development and response to injury (Kingsley, 1994; Hogan, 1996). BMPs are known conserved mediators of retrograde neuronal differentiation from Drosophila to vertebrates (Nishi, 2003; Hippenmeyer et al., 2004; Xu and Hall, 2006). Several lines of evidence suggest that BMPs selectively enhance dendritic growth in cultured neurons and appear to improve motor function in focal ischemia (Lein et al., 1995; Carri et al., 1998; Guo et al., 1998; Le Roux et al., 1999, Withers et al., 2000; Yabe et al., 2002). After traumatic injury, the expression of BMPs became apparent in spinal motor neurons as well as in glial cells (Setoguchi et al., 2001 and 2004). Exogenous BMPs could

enhance axonal growth potential in injured adult spinal cord. The elongation/initiation of neurite extension in both CNS and PNS neurons requires BMP signaling (Parikh et al., 2011).

BMPs function by binding to a complex of transmembrane type I and type II receptors, both of which are indispensable for signal transduction (Massague, 1998). BMP signaling is mediated in part through the Smad-mediated pathway. Interestingly, activation of smad1 has been reported to promote axonal regeneration in rats after SCI (Parikh et al, 2011). However, unlike some other growth factors, BMPs work best within a short range and have a short half-life of 10-30 min. BMPs bind avidly to extracellular matrix proteins and endogenous inhibitors that limit their availability (Hall and Miller, 2004). Therefore, higher amounts of BMP7 may be required to obtain equivalent efficacy. We had employed adenoviral BMP7 gene transfer to continuously augment BMP7 synthesis in cultured neurons and in sciatic nerve injury rat models and demonstrated neuroprotective BMP7 transduction in cultures and *in vivo* (Tsai et al., 2007 & 2010). This study aims to examine whether BMP7 overexpression is able to protect the spinal cord neurons against toxin challenge or contusive injury. Here we show that Ad-BMP7 transduction effectively reduces oxidative/inflammatory cell damage in spinal neuron-glial cultures. *In vivo*, enhanced BMP7 expression significantly improved hindlimb functional recovery in Ad-BMP7-transduced SCI rats.

MATERIALS AND METHODS

Recombinant Adenovirus. BMP7 cDNA was a gift from Dr. Barry Hoeffer (National Institute on Drug Abuse, National Institute of Health, Baltimore, Maryland, USA). Recombinant adenovirus (Ad) encoding BMP7 (Ad-BMP7) was made by Dr. SK Shyue (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). We used first generation E1-deleted adenoviral vector (~ 10^{10} pfu/mL) encoding GFP or BMP7 under the control of PGK (phosphoglycerate kinase) promoter as described previously (Tsai et al., 2007 and 2010a). Briefly, full-length cDNA of human BMP7 or GFP, as a vector control, was cloned into an Ad-shuttle vector. Recombinant adenovirus carrying BMP7 or GFP (A-dGFP) was constructed by homologous recombination in human embryonic kidney (HEK) 293 cells (American Type Culture Collection), as described previously (Liou et al., 2000; Shyue et al., 2001; Yang et al., 2008).The adenovirus with no insert (Ad-PGK) was also used as vector control. Recombinant adenoviruses were purified to a high concentration using a 2-step cesium chloride ultracentrifugation. The viral titers, expressed as a plaque-forming unit (pfu), of the purified recombinant adenovirus were further determined by a plaque-forming assay.

Mixed Glial Cultures. Mixed glial cell cultures were prepared from the cerebral cortexes of newborn Sprague-Dawley (SD) rats as described by Tsai and Lee (1994 & 1998). Briefly, triturated cortexes, free of meninges, were passed through nylon cloths (<80 um) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS). The cells were incubated at 37^{o}C in a water saturated atmosphere of 5% CO_2/95% air. To free glial cultures from contaminated cells, confluent cultures were purified by shaking at 180 rpm to remove the suspended cells. Cells in the flasks were replated into multiwall plates. Cultures showed greater than 95% positive staining for glial fibrillary acidic protein (rabbit or mouse anti-GFAP, Chemicon, USA), an astroglial marker.

Mixed Neuronal/Glial Culture. A mix of neuron-glial cultures were prepared from spinal cords of embryonic SD rat fetuses (gestation days 14-16) as described in previous studies (Hung et al., 2000;Tsai et al., 2005 & 2010b). Briefly, fetal spinal cords were dissociated with a mixed enzymatic solution of papain/protease/ deoxyribonuclease I (0.1%:0.1%:0.03%) and plated onto poly-lysine coated dishes or multiwell plates at density $3x10^5$ cells/cm^2. Cultures were maintained in DMEM supplemented with N2 (Gibco, for serum free condition) or with 10% FBS. Cultures were infected with Ad-GFP (as mock control) or Ad-BMP7 the second day after seeding. Mixed neuron/glial cultures were harvested 2–7 days after adenoviral infections.

Transducing Cultures with Adenoviruses. Ten~fifty multiplicities of infection (MOI) each of Ad-GFP or Ad-BMP7 was added to cultured cells. Two days after infection, cells were harvested for RT-PCR or western blot analysis, and the medium was collected for BMP7 protein detection. To examine the function of overexpressing BMP7, cells were challenged with toxins at two days after adenoviral infection. Two days later, cultured cells were either fixed by 4% paraformaldehyde for morphologic examination or harvested for biochemical assays.

Menadione or Lipopolysaccharide (LPS) Exposure. Non-treated control, Ad-PGK-, or Ad-BMP7-transduced cells were maintained in a serum-free medium and treated with menadione (10uM), a strong oxidizing agent that can generate a high level of ROS (Lamson and Plaza, 2003). One day later, the surviving cells were analyzed for MTT reduction or for neuronal marker staining. In the experiments of LPS exposure, non-treated, Ad-PGK-, or Ad-BMP7-transduced cells were maintained in a serum-containing medium (DMEM + 10% FBS). During 2 days of LPS (100 ~1000 ng/ml) treatment, the culture medium was switched to DMEM + 2% FBS + 2% horse serum. The medium was saved for nitrite assay, while cells were fixed for staining of iNOS and cell markers.

Spinal Cord Injury and Treatment: Adult SD rats weighing 200-275g were used. Spinal cord injury (SCI) was elicited by dropping a 10g rod from a height of 25 mm to the thoracic level 9th segment using a NYU weight-drop impactor as described previously (Cheng et al, 2002;.Huang et al., 2011). Control (sham) animals received only a laminectomy, exposing the cord without disturbing the dura. Antibiotics (Borgal) were given (42 mg) immediately before surgery and daily for 1 week after. Rats were anesthetized with isoflurane. An area extending approximately 4.5 cm above and below the injury site was shaved and disinfected with iodine. A 7.0 cm incision was made over the vertebral column. Next, two incisions were made on either side of the vertebral column, extending about 3.0 cm rostral and caudal to the T9-T10 segment. The vertebrae dorsal and medial to T9-T10 were then cleared and the spinal tissue exposed. A moderate injury was produced by allowing the 10-g impactor to drop 25 mm. Within 30 min after contusive injury, the rats received Ad injection (Fig. 6). A 5 μl Exmire microsyringe with a 31-gauge needle was positioned near the midline of the cords 1 mm rostral and caudal to the contusive center. Ad-GFP (10^7 pfu/animal) or Ad-BMP7 (10^7 pfu/animal) was injected into the dorsal column of the spinal cord (~1 mm ventral to the dura surface). During surgery, the rectal temperature was maintained at 37 ± 0.5 °C using a thermostatically regulated heating pad. Following injury and treatment, the incision was closed and sutured. Each rat was then returned to its cage. To avoid urinary tract infections, manual emptying of the urinary bladder was carried out twice daily. It was noted that experimental animals receiving the adenoviral injection survived well within the entire

experimental period. All surgical interventions and animal care were performed in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, National Institutes of Health and were approved by the Animals Committee of Taipei Veterans General Hospital.

Behavioural Assessment. Rats were allowed to recover for 2, 4, 7, 14 and 28 days post-injury. This time course allowed for examining the effect of BMP7 during the initial deficit in the behavioral locomotor assay and during the plateau phase of recovery after trauma. Recovery of hindlimb stepping was assessed while subjects moved freely about an open field. The Basso, Beattie and Bresnehan (BBB) open field score was used to evaluate locomotion in terms of hindlimb functional improvement of the SCI rats (Basso et al., 1995&1996). The first behavioral assessment was conducted 24 hrs after surgery, and prior to shock treatment. The BBB scale ranges from 0 (no hindlimb movement) to 21 (normal movement-coordinated gait). In this study, behavior analyses of SCI rats were conducted weekly until 4 weeks (N=5 per group). The behavior tests were recorded by a video camera, and two examiners were blind to each behavior evaluation group.

Western Blot Analysis. A conditioned medium of mixed glial cultures was collected for BMP7 detection. After cultured cells were washed twice with PBS, they were solubilized in a lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, pH7.5, 1x protease inhibitors (Roche, Mannheim, Germany), 1 mM PMSF, 1 mM Na3VO4 and 1 mM DTT. Protein concentration of the resultant lysate was determined using the method of Bradford (Bio-Rad protein assay, Bio-Rad, CA, USA). Equal amounts of proteins were loaded and separated using gel electrophoresis (SDS-PAGE) as described previously (Tsai et al., 2005).

Total RNA Extraction and RT-PCR. Total RNAs from mixed glial cultures were prepared using a High Pure RNA isolation kit from Roche (Germany). A Titan one tube RT-PCR kit (Roche) was further used for amplification. The nucleotide sequences of the primers were based on published cDNA sequences of human BMP7 (forward, 50-CATGCTGGA CCTGTACAACGC-30; reverse, 50-CCTCACAGTAGTAGGCG GCG-30). After amplification, the products were separated on 1% agarose gels containing ethidium bromide. The bands were then visualized under ultraviolet (UV) transillumination.

Immunohistochemical Analysis. Primary cultured cells were fixed in 4% paraformaldehyde solution for 30 min. Cells were then permeabilized with 0.2% Triton X-100. Cells were stained with primary antibodies and with the respective fluorescently tagged secondary antibodies (Jackson ImmunoResearch Inc.). Images of cultured cells were obtained with a fluorescent microscope equipped with fluorescence optics. Images of immunoreactive (IR) cells were taken with a CCD camera. IR cell numbers were quantified by blind counting of 10 adjacent microscopic fields at x100 magnification, in each of three wells per condition. Experiments were repeated 3–4 times.

Statistical Analysis. One-way ANOVA followed by Fisher's protected least-significant difference was used to determine statistical differences between treatments *in vivo*. A statistically significant difference was accepted at $p<0.05$.

RESULTS

BMP7 mRNA and Protein Expression in Mixed Glial Cells. After obtaining purified Ad-BMP7, we first aimed to overexpress BMP7 in mixed glial cells. Approximately 10^6 pfu of Ad-BMP7 or Ad-PGK, as a vector control, were added to cultures. Cultures were harvested for reverse transcriptase (RT)-PCR and western blot analysis at 2 days after Ads infection. Higher levels of human-specific BMP7 mRNA could be detected in Ad-BMP7-transduced cultures, while human-specific BMP7 mRNA was not found in Ad-PGK-infected cells (Figure 1A). The predicted sizes of amplicon for BMP7 and beta-actin were 836 and 360 bp, respectively. Figure 1B shows the western blot analysis of cell lysate and conditioned medium from Ad-PGK- and Ad-BMP7-transduced cultures. BMP7 monomer (~17 kDa) was detected in cell lysate, whereas BMP7 dimer (~34 kDa) was found in conditioned medium. Transduction of cultures with BMP7-encoding Ad vector resulted in higher expression levels of BMP7 mRNA and protein. Mixed glial cells were further treated with endotoxin LPS (1 μg/ml). The cells/medium were harvested for nitrite release and western blot analysis 2 days after treatment. As shown in Figure 2A, Ad-BMP7 reduced LPS-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in cultures. Consistent with the result of iNOS expression, nitrite release to the medium was increased significantly by LPS treatment (Figure 2B). Ad-BMP7 transduction ameliorated LPS-induced nitrite release ($P<0.05$, compared to Ad-PGK+LPS).

BMP7 Increased Neuronal Survival and Protected Against Neuronal Death Induced by Oxidative Stress and LPS. The effect of BMP7 overexpression in spinal mixed neuron/glial cultures was examined under two stress conditions: (1) lower density cultures (~10^5 cells/cm^2) in a serum-free condition and (2) higher density cultures (~3 x10^5 cells/cm^2) challenged with menadione, a strong oxidizing agent that can generate a great quantity of ROS when it enters cells (Lamson and Plaza, 2003). In lower density cultures, Ad-BMP7 preserved betaIII tubulin (+) neurons effectively after 5 days in cultures (36443 ± 4607 cells/cm^2 compared to 2555 ± 538 cells/cm^2 in Ad-PGK-transduced cells) as shown in Figure 3 (A & B). For menadione-challenged condition, cultures were seeded at a higher density and transduced with Ad-BMP7 or Ad-PGK on the second day after seeding in a serum-free condition. The following day, cultures were challenged with menadione (10 μM). Cell viability was assessed by MTT assay. When compared to the control, 10 μM menadione applied for 24 h decreased the cell viability by about 36% (Figure 3E). Pre-transduction of spinal cultured neurons with Ad-BMP7 prior to menadione exposure significantly increased cell survival ($P<0.05$, compared to Ad-PGK). For the anti-inflammatory experiment, Ad-PGK- or Ad-BMP7-transduced cultures were grown in a serum-containing medium. During 2 days of LPS treatment, the medium was switched to DMEM+2%FBS+2% horse serum. Ad-PGK- or Ad-BMP7-transduced cells had little iNOS expression (data not shown). Ad-PGK+LPS-treated cultures prominently increased iNOS (+) cells (Figure 3C). Ad-BMP7 transduction significantly reduced LPS-induced iNOS (+) numbers ($P<0.05$, compared to Ad-PGK+LPS shown in Figure 3 (D & F). Concurrently, LPS-induced an increased release of nitrite level in a cultured medium that was attenuated by Ad-BMP7 transduction ($P<0.05$, compared to Ad-PGK+LPS in Figure 3G).

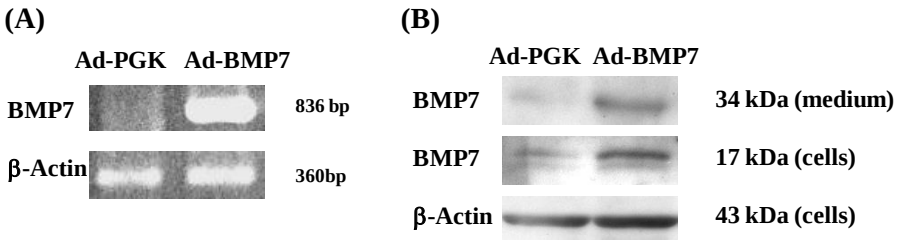

Figure 1. Detection of RNA and protein after Ad-BMP7-mediated gene transfer in cultured glial cells: (A) mRNA expression. Transduction of cells with adenoviral vector encoding BMP7 resulted in higher BMP7 mRNA expression (381 bp). No detectable human specific BMP7 mRNA was found in Ad-PGK-infected tissues. (B) The protein expression. Elevated levels of the BMP7 dimer (~34 kDa) and monomer (~17 kDa) were found in Ad-BMP7-transduced cells. Cultured glial cells were infected with Ad-BMP7 for 48 hrs.

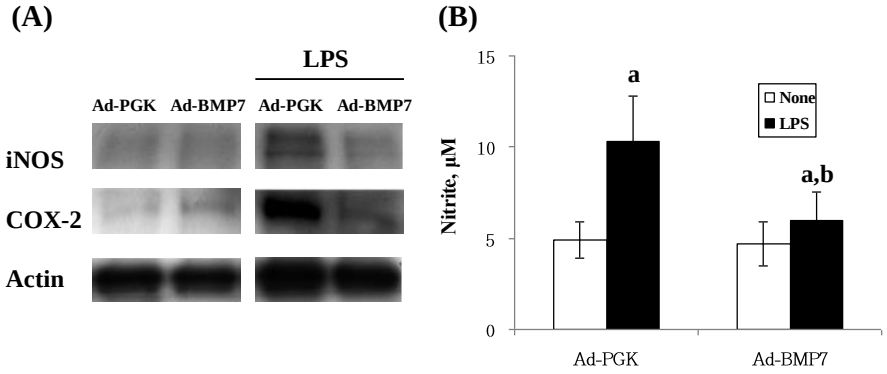

Figure 2. Analysis for effects of Ad-BMP7 transduction on LPS stimulation in cultured glial cells: (A) iNOS and COX-2 protein expression (B) Nitrite release to cultured medium. a $P<0.05$, none vs LPS in Ad-PGK-or Ad-BMP7-transduced cells; b $P<0.05$, Ad-BMP7 compared to Ad-PGK or Ad-BMP7+LPS compared to Ad-PGK+LPS.

Infective Tropism and Transgene Expression of Adenovirus in Spinal Cords. We performed double-label experiments for Ad-GFP-infected tissues, including the thoracic spinal cords level 9 (T9, injection site), and neuronal cell bodies of cortical spinal, rubrospinal and vestibular tracks. One week after injection of Ad-GFP into the spinal cord (T9), GFP was highly expressed at the injection site, and then tapered down rostral-caudally as shown in Figure 4 (A-C). GFP-IR was detected in transverse and longitudinal sections of the spinal cord and the remote neuronal cell bodies of spinal tracks, including the motor cortex, red nucleic and vestibular nuclei. It is noted that Ad-GFP infected into the spinal cord could retrogradely transport to the cell body. To characterize the cell types that expressed GFP at the injection site, we performed immunofluorescence staining with cell-specific markers. Neurons were identified by NeuN (Figure 4J), astrocytes by GFAP (Figure 4L), and progenitor by nestin (Figure 4K). At the injection site, GFP was co-localized with neuron, astroglia and progenitor cells. Interestingly, ependymal cells lining the central canal expressed GFP at the injection site (Figure 4B).

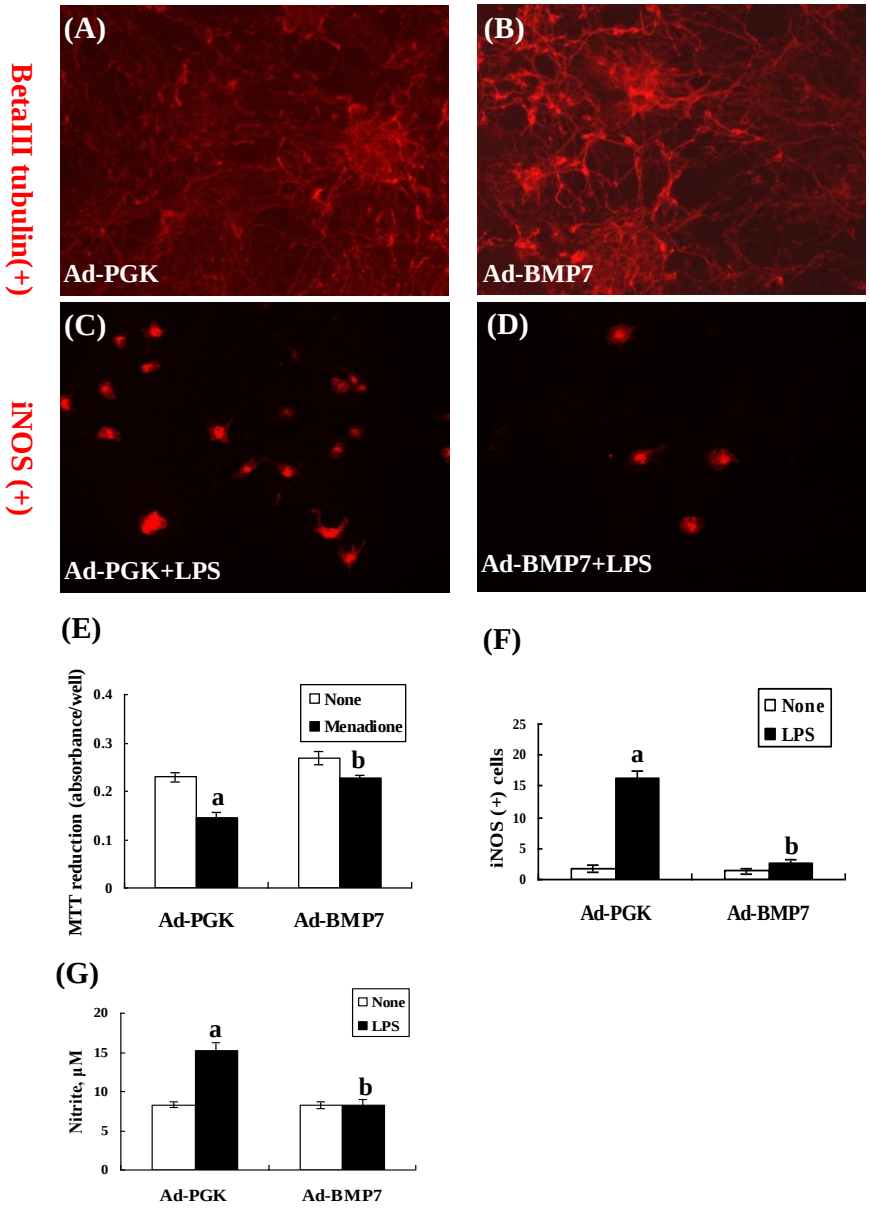

Figure 3. Enhanced BMP7 expression reduced LPS stimulation and menadione toxicity in spinal neuron/glial cells. (A) Ad-PGK-transduced, neuronal marker; (B) Ad-BMP7-transduced, neuronal marker; (C) Ad-BMP7+LPS-treated, iNOS-IR; (D) Ad-PGK+LPS-treated, iNOS-IR, (E) MTT reduction in menadione-treated or non-treated cultures, (F) iNOS (+) cells in LPS-treated on non-treated cultures (G) nitrite release to the medium of LPS-treated or non-treated cultures. Mixed spinal neuron/glial cells were transduced with Ad-PGK or Ad-BMP7. Ad-transduced cells were then challenged with menadione for 1 day or with LPS for 2 days. Enhanced BMP7 expression increased neuronal survival. bIII tubulin (+) cells in (A) Ad-PGK-transduced cells were 2555±538 cells/cm2 and in (B) Ad-BMP7-transduced cells were 36443±4607 cells/cm2.a $P<0.05$, none vs Menadione or LPS in Ad-PGK-or Ad-BMP7-transduced cells; b $P<0.05$, Ad-BMP7 compared to Ad-PGK or Ad-BMP7+Menadione (or LPS) compare to Ad-PGK+Menadione (or LPS).

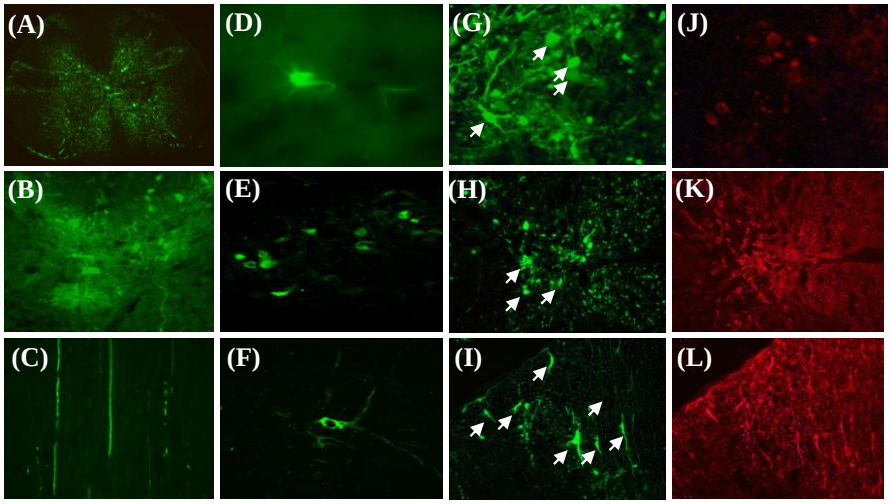

Figure 4. Infective tropism of adenovirus encoding GFP (Ad-GFP), which was injected into the spinal cord 1 week before. The viral construct of Ad-GFP was injected into the thoracic spinal cord, level 9 (T9). After 1 week, fluorescence was detected in transverse and longitudinal sections of the spinal cord, and neuronal cell bodies of corticospinal, rubrospinal and vestibular tracks. (A) GFP-IR cells in T9 cross section; (B) GFP-IR cells near central canal and dorsal funiculus in T9 cross section; (C) GFP-IR cells in longitudinal section of T9-10; (D) GFP-IR cells in motor cortex; (E) GFP-IR cells in red nuclei; (F) GFP-IR cells in vestibular nuclei; (G)~(L) are cells in T9 cross section; (G) GFP-IR cells double stained with NeuN (Figure 4J); (H) GFP-IR cells double stained with nestin (Figure 4K); (I) GFP-IR cells double stained with GFAP (Figure 4L).

Figure 5 shows the prolonged GFP expression at 2 and 4 weeks after Ad-GFP injection into the spinal cord. Strong GFP expressions were found at 2 weeks post-infection, although GFP expression slightly decreased at 4 weeks post-injection, as shown in Figure 5 (A & B).

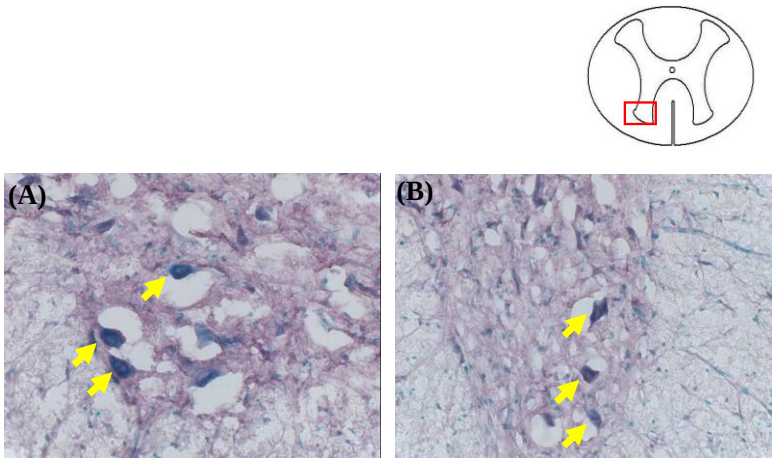

Figure 5. Duration of transgene expression after infection of Ad-GFP to the spinal cords: (A) GFP-IR in cross section of spinal cord at 2 weeks after infection. (B) GFP-IR at 4 weeks after infection.

BMP7 Expression in Injured Spinal Cords after Ad-BMP7 Transduction. Ad-BMP7 was introduced to contused spinal cords following the same procedure as the Ad-GFP injection (Figure 6A). Western blot analysis was conducted at 3 days post-transduction to determine adenovirus mediated BMP7 expression *in vivo*. As shown in Figure 6B, BMP7 protein expression was not detectable in sham control spinal cords. However, BMP7 expression was readily detected in Ad-GFP-infected spinal cords of SCI rats at 3 days post-injury. BMP7 expression was further increased in spinal cords of SCI rats receiving Ad-BMP7 transduction. Concurrently, immunoreactivity (IR) of growth-associated protein 43 (GAP-43), a marker for active axon growth, was more preserved in Ad-BMP7-transduced cords (Figure 6D) than in Ad-GFP-transduced cords (Figure 6C).

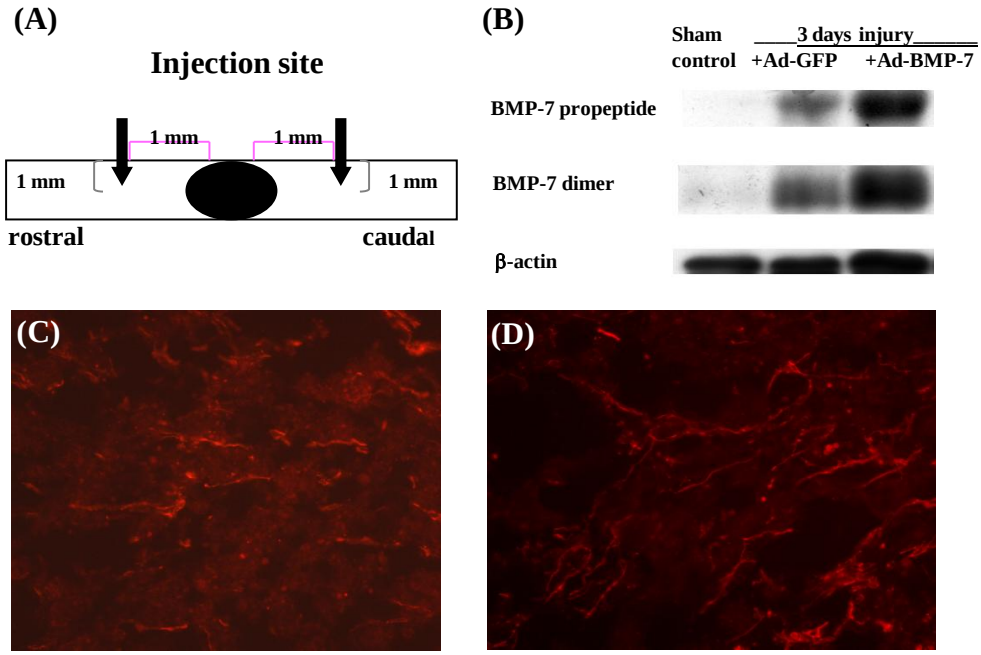

Figure 6. Ad-BMP7 infection to contusive spinal cord further increased BMP7 expression and preserved more spinal fibers in SCI rats. Adenovirus-mediated BMP7 expression in contused spinal cords at three days post-injury: (A) Ad-GFP or Ad-BMP7 at a dose of 1x10^7 plaque forming units (pfu) per rat was administrated into the dorsal column of spinal cords at 1mm rostral or caudal to the contusive center. (B) BMP7 expression in contused spinal cord epicenter. (C) GAP43-IR in Ad-GFP-transduced spinal cord; (D) GAP43-IR in Ad-BMP7-transduced spinal cords.

BMP7 Gene Delivery Improved Locomotion in SCI Rats. The hindlimb motor function of SCI rats receiving various treatments was monitored using the guideline of BBB Locomotor Rating Scale (Basso et al., 1995 & 1996). The rats receiving Ad-BMP7 started to show partial weigh support with their hindlimbs at 14 days after SCI, when compared to the control groups that underwent the same procedure, but received no treatment or the control Ad-GFP vector (Figure 7). Moreover, statistically significant differences in BBB scores ($P < 0.05$) between Ad-BMP7-treated rats and control rats were found at 21 and 28 days after contusion. By the third week partial hindlimb coordination could be found in the Ad-BMP7-

treated rats, whereas most rats in both control groups exhibited either no weight support or hindlimb coordination throughout the entire survival period.

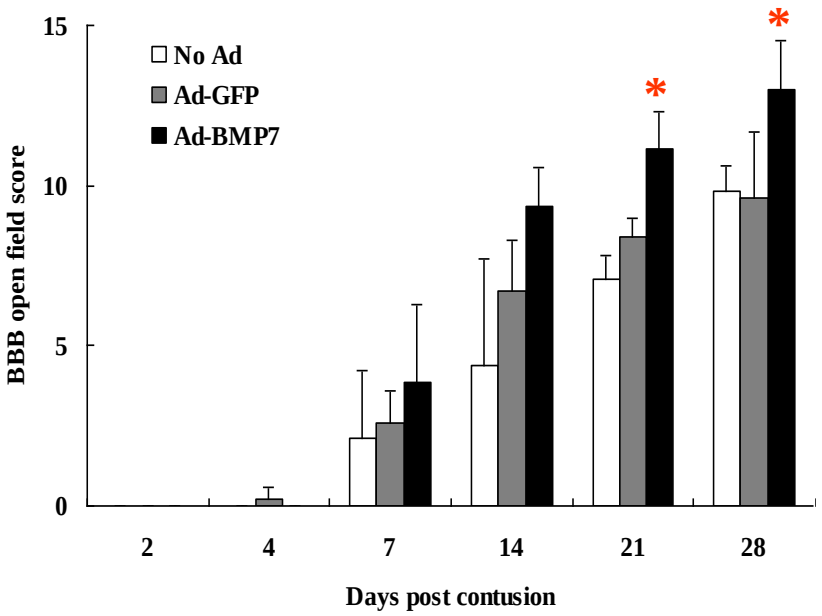

Figure 7. Ad-BMP7 infection to contusive spinal cord promoted functional restoration in SCI rats. Time course of hindlimb locomotor recovery in rats receiving BMP7 gene transfer. Time course of change in BBB open field scores in rats receiving various treatments after contusive injury. Ad-GFP (n=5); Ad-BMP7 (n=5); No Ad (n=5); * P< 0.05 by t-test. Data represent the mean±SEM.

DISCUSSION

The central observation of this study was that BMP7 gene transfer not only alleviated menadione-induced neuronal death and LPS stimulation in spinal cord cultures, but also improved functional recovery after SCI in rats. There were significant differences in BBB scores between Ad-BMP7-treated and Ad-PGK-treated groups, indicating that BMP7 gene transfer induced hindlimb functional restoration in SCI rats. In the present study, recovery in hindlimb locomotor function induced by BMP7 gene transfer appeared to be correlated with the nerve fibers remaining in the injured spinal cord. Accordingly, the effect of BMP7 gene transfer on functional recovery could be due to preventing neurons from oxidative insult and inflammation caused by SCI.

We used first-generation E1-deleted Adenoviral vectors. Although we did not assess immune response in this study, there were no significant side effects in rats with Ad injection. Ad-BMP7 or Ad-GFP used in this study were constructed with a phosphoglycerate kinase (PGK) promoter that could drive prolonged (days to weeks) transgene expression. As a result, prominent GFP expression was observed in the spinal cord up to one month after infection. At the injection site, different cell types including neurons, astrocytes, progenitors and ependymal cells expressed transgene GFP. These results are similar to two previous studies (Wang et al., 2011; Abdellatif et al., 2006). The infective tropism of Ad-GFP showed that

GFP-transduced cells were not limited to the injected site. Extensive GFP expression was observed in the remote neuronal cell bodies, including motor cortex, red nuclei and vestibular nuclei etc. This indicates that Ad-GFP was internalized by the spinal nerve fiber and retrogradely transported to the somata of long projection axons. Thus, transgene expression can be achieved in the spinal cords and the corresponding neuronal cell bodies after injection of Ad-GFP (or Ad-BMP7) into the spinal cords.

Based on RT-PCR and Western blot analysis, no BMP7 expression was observed in the spinal cord of Ad-treated rats. Yet, contusive injury induced an increase of BMP7 expression in spinal cords. Intraspinal administration of Ad-BMP7 further increased BMP7 expression level. This contused spinal cord may serve as a reservoir for BMP7, supporting neuronal survival post-injection. BMP7 expression in the contused spinal cord at later time points post-injury was not characterized in Ad-BMP7-treated rats. However, investigation via injection of Ad-GFP at a similar pfu and condition to that of Ad-BMP7 indicates that GFP expression can last for at least 4 weeks (Figure 5.). This observation may provide information addressing the possible expression duration of Ad-BMP7 gene transfer to the contused spinal cord.

BMPs are synthesized as precursor proteins, then are proteolytically cleaved at the N-terminus and secreted into the extracellular matrix. BMPs dimerize through disulfide bonds and initiate signaling by binding cooperatively to both Type I and Type II transmembrane serine/threonine kinase receptors (BMPRI and BMPRII) (Massague, 1998). The biological activity of BMP is determined by the levels of BMP ligands and receptors, as well as by that of soluble BMP antagonists. In addition, BMPs bind avidly to extracellular matrix proteins, both of which may limit their diffusion through tissues. Through Ad gene transfer of BMP7 to culture cells and spinal cords *in vivo*, biologically active BMP7 could have been produced continuously inside cells and released extra-cellularly in the present study. We have been able to show enhanced expression of BMP7, either in the dimer or the proform in Ad-BMP7-infected nerves or injured nerves (Figure 1 & 6B). This is consistent with the results of Setoguchi et al (2001 & 2004) who showed an increased expression of BMP2 and BMP7 in the adult spinal cord after injury.

Production of reactive oxygen species (ROS) by oxidative stress can modify DNA, proteins, lipids and carbohydrates in mammalian cells (Floyd 1999). Spinal cord neurons are highly susceptible to ROS. Menadione is a strong oxidizing agent that can generate high levels of ROS when it enters cells (Lamson and Plaza 2003). Accordingly, Ad-BMP7 transduction in spinal cord neurons effectively reduces menadione-induced toxicity. Excessive NO derived from iNOS in LPS-activated cultures is implicated in inflammation and cytotoxicity (Nathan and Xie, 1994). The LPS-induced generation of ROS in microglia/macrophages is an upstream event serving to regulate the production of other pro-inflammatory factors (Sanglioglu et al., 2001; Hsu and Wen, 2002). In the present study, Ad-BMP7 transduction in spinal cord neurons and glial cells effectively reduced LPS stimulation. Our data demonstrate that BMP7 has anti-oxidant and anti-inflammatory effects on spinal neurons and suggests a heretofore unappreciated therapeutic potential for BMP7 in SCIs that are characterized by oxidant and inflammatory stress.

CONCLUSION

In summary, the present study demonstrated that the beneficial effect of BMP7 gene transfer on neuronal survival and axonal regeneration may foster injured rats to recover from SCI. Low-dose and continuously expressed BMP7 by adenovirus-mediated transfer is neuroprotective. Our results suggest that gene transfer may represent a more effective approach to neuroprotection. These studies suggest that BMP7 overexpression has beneficial effects against CNS injury. Future studies using electrophysiological measurement of somatosensory/motor-evoked potential (SSEPs/MEPs) may provide further insights into the recovery mechanism of BMP7 gene therapy.

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