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Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a promising new therapeutic for spinal fusion procedures. Currently, rhBMP-2 is used "off-label" for spinal fusion in cases where there is concomitant spinal cord injury (SCI), yet little is known about the direct effects of rhBMP-2 on the recovery from a SCI. We therefore performed a series of studies in rats to determine whether rhBMP-2, used for spinal fusion, could penetrate the injured spinal cord. Additionally, we sought to determine whether the use of rhBMP-2 led to morphologic changes within the injured spinal cord that may alter functional recovery from SCI.

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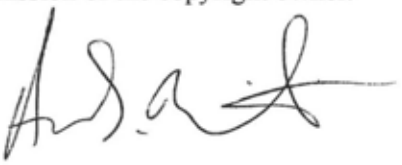

Anton E. Dmitriev

Neuroscience Graduate Program

Uniformed Services of the Health Sciences

DEDICATION

This work is dedicated to my mother Valeria S. Dmitrieva, PhD, an exemplary educator who has always been my role model and believed that everything is possible through hard work and perseverance. Your untimely passing was an irreplaceable loss but I hope you would have been proud of my achievement.

ABSTRACT

The effects or rhBMP-2 used for spinal fusion on spinal cord pathology after traumatic injury

by

Anton E. Dmitriev

Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a promising new therapeutic for spinal fusion procedures. Currently, rhBMP-2 is used “off-label” for spinal fusion in cases where there is concomitant spinal cord injury (SCI), yet little is known about the direct effects of rhBMP-2 on the recovery from a SCI. We therefore performed a series of studies in rats to determine whether rhBMP-2, used for spinal fusion, could penetrate the injured spinal cord. Additionally, we sought to determine whether the use of rhBMP-2 led to morphologic changes within the injured spinal cord that may alter functional recovery from SCI.

In the first study we observed functional BMP signaling within the spinal cord when rhBMP-2 was implanted on the spinal column at different times after a dorsal hemisection. BMP-specific signaling, indicated by phosphoSmad 1,5,8 (pSmad) immunohistochemisty, was observed in neurons, glia, macrophages and fibroblasts in the spinal cord. Increased pSmad labeling around the spinal cord lesion in the rhBMP-2 group directly correlated with increased permeability of the blood-spinal cord barrier at specific times following SCI, assessed by intravenous injection of luciferase.

A second set of experiments examined the morphological and functional effects of implanting rhBMP-2 on the spinal column at the level of a dorsal hemisection SCI. By

one week post-lesion, rhBMP-2 treatment significantly increased the inflammatory response to injury as compared to controls. In addition, there were elevated levels of reactive astrocytes, infiltrating fibroblasts and inhibitory proteoglycans around the lesion in rats treated with rhBMP-2 relative to control animals. These differences persisted six weeks following injury. Functional tests of locomotor activity (BBB and footprint analysis) revealed significant deficits in animals treated with rhBMP-2 at one week post lesion and some residual deficits remaining six weeks after lesion relative to control animals. Collectively, these data demonstrate that rhBMP-2 used for spinal fusion reduces recovery from a concomitant SCI.

The results from our studies indicate that clinical use of rhBMP-2 in the vicinity of a SCI may have detrimental effects on neurologic recovery.

THE EFFECTS OF rhBMP-2 USED FOR SPINAL FUSION ON SPINAL CORD PATHOLOGY AFTER TRAUMATIC INJURY

by

Anton E. Dmitriev

Doctoral Dissertation submitted to the faculty of the Graduate Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Thesis directed by:
Aviva J. Symes, Ph.D.
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Standing at the end of a long road of formal education certainly gives me a sense of measurable accomplishment. At the same time my academic endeavors are far from over and I do feel that now I am entering a new stage of development as a professional scientist.

Having reached this point in my career, I am honored and humbled to admit that none of this would have been possible without the constant support, advice and guidance of those around me. I have to give special thanks to my immediate advisor, Dr. Aviva Symes, for her limitless availability, academic guidance and critical reviews of my Russian-style writing. During the past 18 months, despite giving birth to her son, Eli, and overcoming the battle with skin cancer, she never said “no” or “I don’t have time” to anything I approached her with. And for this I am grateful. I am also sincerely thankful for keeping me on track and allowing me to finish in a timely fashion.

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Dr. Ronald Lehman, Jr. has provided me with vital information on the clinical relevance of my project. I would like to thank him for being supportive and understanding and hope that the Outstanding Paper Award from the North American

Spine Society that our project won this year is a good indication that I wasn't wasting too much time while being away from WRAMC.

I would also like to acknowledge Dr. Juanita Anders and members of her lab, particularly, Xingjia Wu. My rotation in your lab has given me a great foundation in spinal cord injury research that became the basis for my studies. Thank you for your advice and support and thank you, Xingjia, for being patient with all my pesky questions.

I would like to extend my sincere gratitude to Dr. Gregory Mueller, my committee chair, for his support and flexibility. Thank you for making sure that my committee meetings went smoothly and on time.

It would be unfair if I did not acknowledge the wonderful members of the Symes lab: Dr. Bala Susarla, Suzanne Farhang and Eric Laing. Bala, you've taught me all the techniques in the molecular biology and I want to thank you for that. I have to give special thanks to Suzie Farhang whose technical skills, hard work and dedication to the project have facilitated my timely data collection. And Eric, thanks for your help on the new DRG project and I am excited that you'll be joining the USUHS graduate student community in the near future.

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CHAPTER 1

Introduction

Spinal cord trauma remains one of the most devastating types of injury with no effective treatment and / or cure. It is estimated that there are over 2.5 million people living with spinal cord injury (SCI) worldwide and over 130,000 new trauma cases occur each year (Thuret et al., 2006). Within the United States this figure approaches 200,000 people and approximately an additional 10,000 people suffer the injury annually.

Neurobiology of SCI

Cellular and biochemical changes following a SCI

Spinal cord injury consists of two widely accepted stages: primary mechanical insult and secondary injury resulting from a cascade of biophysical and neurochemical changes in the residual tissue surrounding the site of trauma. Primary injury can result from a contusion, which is the most common type of injury, compression or penetration of the spinal cord. These insults lead to an immediate cell death including neurons, oligodendrocytes, and astrocytes (Hagg and Oudega, 2006). Secondary injury contributes to further cellular loss through hemorrhage, ischemia, excitotoxicity, inflammatory response and oligodendrocyte apoptosis with concomitant demyelination of surviving axons (Thuret et al., 2006). Following a SCI is the formation of a glial scar that demarcates the injury zone. Glial scarring is facilitated by the surviving astrocytes, which become reactive, glial progenitors, oligodendrocyte progenitor cells (OPCs), invading meningeal fibroblasts and macrophages and activated microglia (Fawcett, 2006). During gliosis the above cells secrete chondroitin sulfate proteoglycans (CSPGs)

in the extracellular matrix. CSPGs consist of a protein core linked by four sugar moieties to a sulfated glycosaminoglycan (GAG) chain that contains repeating disaccharide unit (Galtrey and Fawcett, 2007). They comprise a large family of molecules to include aggrecan, brevican, neurocan, NG2, phosphacan and versican, which share chondroitin sulfate side chains and differ in the protein cores and the extent of side chain sulfation (Morgenstern et al., 2002). These molecules are important in establishing developmental boundaries and have been associated with inhibition of neuronal growth both *in vitro* and *in vivo*. CSPG expression is minimal in the intact spinal cord; however, is upregulated following an injury and formation of the glial scar (Miller and Silver, 2006).

Changes in the blood spinal cord barrier (BSCB) following a SCI

In addition, spinal cord trauma results in the disruption of the meningeal and the blood spinal cord barriers (BSCB), which isolate the spinal cord from the surrounding tissues and allow for a selective molecule exchange between the neural tissues and local microvasculature (Nicholas and Weller, 1988; Vandenabeele et al., 1996).

The meninges enclosing the central nervous system (CNS) constitute a virtually impermeable barrier between the CNS and the surrounding tissues. Morphologically, three distinct meningeal layers have been described: the outermost dura mater, the intermediate arachnoid mater and the innermost pia mater (Vandenabeele et al., 1996). The physiologic meningeal barrier is comprised within the arachnoid barrier cell layer, which is characterized with the presence of a continuous basal lamina, numerous desmosomes, tight and gap junctions and a lack of large extracellular spaces (Vandenabeele et al., 1996). These features of the arachnoid mater represent an effective

morphological and functional meningeal barrier between the circulating blood of the outer dura and the cerebrospinal fluid filling the subarachnoid space (Haines et al., 1993). In contrast, the outermost dura mater layer of spinal meninges lacks the tight junctions and serves primarily as an elastic protective membrane that can accommodate stretching and deformation experienced during spinal movement and postural changes. These properties of the outermost dura are explained by the presence of elastic fibers and helically interwoven extracellular collagen that provide additional tensile strength to the dural membrane (Kumar et al., 1996). Within the spinal cord proper, the blood spinal cord barrier (BSCB) between internal microvasculature and the surrounding neural tissues is composed of the tight junctions connecting endothelial cells as well as the astrocytic end-feet that ensheath vessels and interact with the vascular pericytes (Nicholas and Weller, 1988).

Increased BSCB permeability has been reported in both the spinal cord contusion and hemisection injury models (Noble and Wrathall, 1987, 1988; Maikos and Shreiber, 2007). The initial physical insult results in the mechanical disruption of the meningeal barrier and the vascular BSCB with the extent dependent on the severity of the injury (Maikos and Shreiber, 2007). This leads to an immediate influx of a variety of molecules that are normally excluded from the spinal cord tissues that can range in size from small molecules, after a minor disruption, to red blood cells following a gross hemorrhage (Popovich et al., 1996). Secondary injury events, including cellular apoptosis and necrosis contribute to a more widespread degradation of the BSCB at areas surrounding the lesion over an extended time frame (Mautes et al., 2000).

Studies looking at the post-injury permeability of the BSCB indicate that the barrier reforms approximately 14-21 days after injury, depending on the severity of the insult, the animal model and the type of tracer used to study BSCB (Noble and Maxwell, 1983; Noble and Wrathall, 1988; Popovich et al., 1996; Jaeger and Blight, 1997; Whetstone et al., 2003; Maikos and Shreiber, 2007). The greatest disruption of the BSCB occurs a few hours after the injury; however, a secondary peak of barrier permeability falls between the 3rd and 7th day post-injury and is associated with the increased revascularization of the spinal cord (Whetstone et al., 2003). Common tracers used to study the BSCB integrity include luciferase, a 61kDa protein, and horseradish peroxidase, a 40kDa protein (Jaeger and Blight, 1997; Whetstone et al., 2003; Sharma, 2005). Additionally, albumin extravasation within the spinal cord parenchyma has been used as a marker for BSCB permeability (Gordh et al., 2006). Intravenously injected luciferase has been shown to infiltrate the mouse spinal cord until 21 days after injury (Whetstone et al., 2003), whereas horseradish peroxidase has been detected within the guinea pig and rat spinal cord until 14 days post-lesion (Noble and Wrathall, 1989; Jaeger and Blight, 1997).

Clinically, direct dural lacerations are associated with certain mechanisms of spinal trauma, and are particularly predominant in war related injuries (Carl et al., 2000; Kahraman et al., 2004). The rates of coincidental dural tears have been reported in up to 74% of patients following high-energy induced trauma commonly suffered by the military personnel (Bellabarba et al., 2006). Iatrogenic durotomy is another known complication in spine surgery with rates ranging from 1% to 14% (Tafazal and Sell, 2005).

Clinical SCI presentation

Patients being admitted to the emergency department with a suspicion for spine trauma routinely undergo initial neurologic evaluation according to the American Spinal Injury Association (ASIA) impairment scale (AIS), which is a five point system (grades A through E) developed in 1992 (Ho et al., 2007). Grade A indicates complete loss of motor function below the level of the injury and full loss of sensory sensation at the lowest sacral segments (S4/5). Grade B represents an incomplete injury with no motor function preservation; however, retention of sensory input from below the level of the lesion and distal sacral segments. Grade C, in addition to axonal sparing described in Grade B, indicates that at least half of the key muscles below the injury level maintain some functionality (able to move against gravity without additional resistance). Grade D is also indicative of sensory sparing as well as motor function preservation below the lesion site with more than half of the key muscles being able to function against gravity and additional resistance. Finally, Grade E represents normal functionality with or without slight reflex changes (Ho et al., 2007).

Surgical management of SCI patients

Spinal instability ensuing after vertebral column fracture is the primary indicator for surgical intervention and spinal stabilization (Lenoir et al., 2006). Spinal re-alignment is critical in cases with multi-level spinal trauma of high-energy etiology. In addition to spinal cord compression, these are often associated with concomitant meningeal lacerations and cerebrospinal fluid (CSF) leakage, which require surgical repair

(Bellabarba et al., 2006). Timing of the decompressive procedure is critical following a SCI and offers advantages to patients with incomplete injuries if performed within the first 24 hours following the accident (Fehlings and Perrin, 2005). Surgical management of spinal column instability requires internal fixation, together with biologic bone growth extenders and/or substitutes.

Historically, autologous iliac crest bone graft (ICBG) obtained from the patient's hip bone has been used for spinal fusions (Sandhu et al., 1999). Overall, the use of autologous bone has been successful in inducing spinal healing but collection of the bone graft material has been associated with additional morbidity, complications and pain at the donor site (Sawin et al., 1998; Silber et al., 2003). Recently, bone morphogenetic proteins (BMPs), specifically BMP-2 and BMP-7, have been introduced as effective bone graft substitutes with healing rates approaching and/or exceeding those obtained with the autologous bone (Mummaneni et al., 2004; Villavicencio et al., 2005; Hamilton et al., 2008). Clinical use of BMPs also obviates the need for iliac crest resection, thus eliminating the donor site morbidity and complications (Mummaneni et al., 2004). In 2002, the FDA approved the use of the recombinant human BMP-2 (rhBMP-2) for treatment of discogenic pain in the lower lumbar spine (FDA, 2002; Khan and Lane, 2004). Favorable clinical results in this application and improved healing of the appendicular skeleton fractures stimulated increased use of rhBMP-2 "off-label" in the thoracic and cervical spinal regions (Boden et al., 2002; Baskin et al., 2003; Glassman et al., 2007a; Glassman et al., 2007b). More recently, rhBMP-2 has been used for inducing posterolateral fusion in patients with spinal trauma and the concomitant SCI (Personal communication with COL(R) Kuklo, MD).

Spinal column arthrodesis using rhBMP-2 with concomitant SCI

As surgical decompression and fusion following a SCI must be performed within the first 24 hours post-injury, implantation of rhBMP-2 in the vicinity of a lesion will expose the injured spinal cord to the exogenous protein. Clinically, up to 12mg of rhBMP-2 are applied per spinal level over the posterior lamina (cervical spine) or transverse processes (thoracolumbar spine) using absorbable collagen sponges (ACS) as carrier material. This results in a pool of blood/rhBMP-2 solution surrounding the spine (Figure 1). In perspective, it has been estimated that normal bone contains less than 2mg of BMP-2 per kilogram of cortical bone (Walker and Wright, 2002). Thus, rhBMP-2, applied at pharmacologic doses on collagen sponges to the spinal vertebrae has the potential to penetrate the spinal cord after even a minor injury to the meninges and BSCB. Surprisingly, little is known about the direct effects of the exogenous rhBMP-2 on the cells comprising the spinal cord. However, recently a number of clinical reports have been published highlighting post-operative complications that include soft tissue

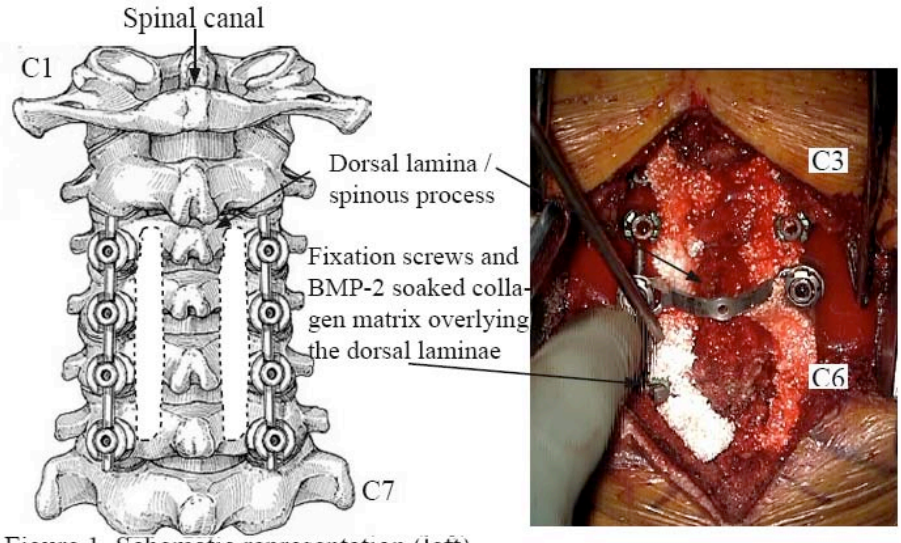

Figure 1. Schematic representation (left) and an intra-operative image (right) of the posterior cervical spine (From Walter Reed Army Medical Center - Jan 2008). Note the BMP-2 soaked collagen matrix (white) placed directly over the spinal canal and surrounded by local blood

swelling, edema, heterotopic bone formation and radiculitis following “off-label” use of rhBMP-2 in treatment of the degenerative spinal disorders (Shields et al., 2006; Joseph and Rampersaud, 2007; Crawford et al., 2009; Rihn et al., 2009). These reports and the paucity of basic science data supporting the safety of rhBMP-2 application around a spinal cord lesion raise concerns with its “off-label” use in patients suffering both the spinal column and cord injuries, despite its proven efficacy in stimulating bone formation.

BMPs, receptors, antagonists and signaling

BMPs were first described by Marshall R. Urist in 1965 as components of demineralized bone matrix that induced connective tissue and cartilage differentiation into bone in extraskeletal locations in the rat (Urist, 1965). Since then, more than 30 BMP family members have been identified, all belonging to the TGF-beta superfamily of cytokines (Riley et al., 1996; Shi and Massague, 2003). Although named BMPs because of their osteoinductive effects noted by Urist, this protein family has now been implicated in many developmental and pathologic processes unrelated to bone formation (Walker and Wright, 2002). BMPs can be subdivided into several groups based on the amino acid sequence and structural similarities: BMP-2, a 26kDa homodimer, is most closely related to BMP-4, and distinct from the BMP-5, -6, and -7 subclass. All BMPs signal through two main types of transmembrane receptors (Types I and II) with specific serine / threonine kinase activity. Type I receptors are the main effector component of the ligand-receptor complex, initiating the intracellular signaling cascade. Upon ligand binding, Type I receptor activation depends on forming a complex with and being directly

phosphorylated by the Type II receptor, which is constitutively active. BMPs bind to three distinct Type I receptors (Activin receptor like kinases (ALK-2, ALK-3 (BMPR-IA) and ALK-6 (BMPR-IB)). BMP-2 selectively binds BMPR-IA and BMPR-IB (Keller et al., 2004). Following Type I receptor phosphorylation BMP signals are transmitted through Smad protein dependent pathways. Smad1, Smad5 and Smad8, also known as receptor-Smads or R-Smads, are directly phosphorylated by the type I BMP receptors and complex with Smad4, a co-Smad. The Smad protein complex then translocates to the nucleus and regulates gene expression (Park, 2005; Goto et al., 2007). Intracellularly, BMP signaling can be inhibited by one of the two inhibitory Smads, Smad6 or Smad7, which compete with R-Smads for Type I receptor interaction (Park, 2005).

Endogenous BMP activity is also subject to a precise extracellular regulation via a number of BMP-specific antagonists that include noggin, follistatin, chordin and gremlin (Balemans and Van Hul, 2002). The majority of these antagonists modulate BMP signaling during development and only noggin and follistatin have been detected in the adult spinal cord (Hall and Miller, 2004; Hampton et al., 2007). Their expression is upregulated after SCI in concordance with that of endogenous BMP-2, -4, and -7 (Setoguchi et al., 2001; Hampton et al., 2007).

BMPs and the spinal cord

In the spinal cord, BMPs play an important role during neurodevelopment as critical regulators of the dorsoventral patterning of the neural tube and neural cell fate determination (Hall and Miller, 2004). BMPs facilitate dorsal cellular identity and oppose / inhibit ventral cell type development stimulated by the sonic hedgehog (Shh) signaling

protein. BMPs may also inhibit oligodendrocyte precursor formation in the developing cord (Ono et al., 1995; Liem et al., 2000; Mekki-Dauriac et al., 2002). In the adult spinal cord, BMP receptors are maintained on neurons and glia and low levels of BMP-2, -4 and -7 have been detected within the intact spinal cord sections (Setoguchi et al., 2001; Setoguchi et al., 2004). Following SCI, expression of BMP-2 and BMP-7 is significantly upregulated (Setoguchi et al., 2004; Fuller et al., 2007) and inhibition of endogenous BMPs by noggin infusion at the site of injury has been shown to improve corticospinal axon regeneration and functional recovery in a rat model (Matsuura et al., 2008). Additionally, BMP expression is increased following a demyelinating lesion, specifically in GFAP-positive reactive astrocytes (Fuller et al., 2007). *In vitro,* BMP-2 has also been implicated in inhibiting neurite outgrowth of the cerebellar granule neurons (Matsuura et al., 2007). However, the overall effects of BMP-2 on neuronal growth are not fully understood, as Zou et al has recently published, in contrast to previous studies, that axotomy induced Smad-1 upregulation increases axonal growth in the adult sensory neurons (Zou et al., 2009).

BMPs play a critical role in cell fate regulation in the adult CNS. BMPs inhibit development of the mature oligodendrocytes from oligodendrocyte precursor cells (OPCs) *in vitro* and *in vivo*, instead encouraging their differentiation into astrocytes (Mabie et al., 1997; Mehler et al., 1997). In addition, neural precursor cells (NPCs) expressing the BMP-specific antagonist, noggin, can be rescued from differentiating into astrocytes following transplantation to the site of a SCI (Setoguchi et al., 2004). These findings indicate that BMPs steer the OPC and NPC differentiation along the astrocytic lineage, which could have significant consequences on the extent of spontaneous

recovery following a SCI. Astrocytes are a major component of the glial scar; therefore, increased numbers of this cell type could facilitate increased scar formation. Glial fibrillary acidic protein (GFAP) is the hallmark sign of reactive astrogliosis and BMP has been shown to promote its expression within this cell type (Dore et al., 2009). Additionally, BMP signaling in astrocytes has been associated with the increased production of inhibitory CSPGs, which limit axonal regeneration through the lesion core (Morgenstern et al., 2002; Fitch and Silver, 2007; Fuller et al., 2007). Furthermore, a recent study by Hong and colleagues demonstrated that addition of BMP-6 to a macrophage culture induced expression of the pro-inflammatory inducible nitric oxide synthase (iNOS) and TNF-α by the cells (Hong et al., 2008). As BMP-2 shares approximately 60% homology in amino acid sequence with BMP-6, it is plausible to assume that rhBMP-2 would also stimulate production of the pro-inflammatory cytokines by macrophages (Rueger, 2002). Increased inflammatory response could in turn contribute to secondary tissue damage and cell loss after the primary insult.

Clinical application of pharmacologic doses of rhBMP-2 to the spinal column in the presence of meningeal defects and a disrupted BSCB is likely to interfere with the course of spontaneous spinal cord recovery following injury. Through increased inflammation, astrogliosis, CSPG deposition, rhBMP-2 could undermine the patients' ultimate potential at regaining functionality. rhBMP-2 is currently being used in patients for spinal fusion and is increasingly indicated for treatment of spinal column instability with concomitant spinal cord pathology. Therefore, these practices necessitate research aimed at expanding our understanding of rhBMP-2 penetration and its direct effects on the cells comprising spinal cord.

Hypothesis: rhBMP-2 used for spinal fusion procedures will penetrate the injured spinal cord and alter the injury response.

Aim 1: Examine penetration of rhBMP-2 into the spinal cord, when applied over the spinal column, at specific time-points following thoracic dorsal hemisection of the rat spinal cord, and correlate it with the reformation of the BSCB. Following a SCI in a rat model, BSCB and meningeal barriers protecting the spinal cord remain porous to exogenous proteins up to 21 days post-lesion. Therefore, we applied rhBMP-2 at several time points between day 0 and 21 after a dorsal hemisection injury and determined the extent of BMP specific signaling activation within the spinal cord using immunohistochemical techniques. BSCB reformation was analyzed using a vascular marker and a correlation between intrathecal signaling activation and barrier permeability was calculated.

Aim 2: Evaluate the post-lesion morphologic changes within the spinal cord associated with rhBMP-2 application to the spinal column and compare these changes with functional outcome. In a clinical setting, post-SCI spinal decompression and fusion yields better outcomes if performed within the first 8 hours after injury. Therefore, in a rat model of dorsal hemisection SCI, we performed spinal arthrodesis with or without rhBMP-2 30 minutes post-lesion. Intrathecal inflammation, gliosis and axonal regeneration were assessed acutely at 7 days post-lesion and in a chronic scenario at 6 weeks post-lesion using immunohistochemical techniques. BBB scoring and

digitized footprint analysis were implemented to assess animals' functional recovery on a weekly basis.

CHAPTER 2

BMP-2 Used in Spinal Fusion with Spinal Cord Injury Penetrates Intrathecally and Elicits a Functional Signaling Cascade

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Ronald A. Lehman, Jr., MD, # Geoffrey SF Ling, MD, PhD ^ and Aviva J. Symes, PhD,§

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Medical Center, Washington, DC.

Running Head: rhBMP-2 Elicits Intrathecal Signaling in the Presence of Spinal Cord Injury

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Abstract

Background Context: The use of recombinant human bone morphogenetic protein - 2 (rhBMP-2) and its indications for spinal fusion continue to be expanded with recent reports citing spinal trauma application. However, there are no data establishing the effects of rhBMP-2 on the injured spinal cord.

Purpose: The purpose of this study was to evaluate the extent of BMP-specific intrathecal signaling following application to the spine at various time-points after a spinal cord injury.

Study Design: This is an *in vivo* rat study using a combination of the dorsal hemisection spinal cord injury and posterolateral arthrodesis animal models.

Methods: Sixty-five female Sprague-Dawley rats underwent either a T9-10 dorsal hemisection SCI (n=52) or laminectomy-only (n=13). SCI animals were further subdivided into 4 follow-up groups (n=13/group): 30min, 24hrs, 7days and 21days, at which time one of two secondary surgeries were performed: 1) Eight rats / time point received either 43μg of rhBMP-2/side or sterile water control over T9-11 on collagen sponges (ACS). Animals were perfused 24hrs after and spinal cords immunohistochemically analyzed. Sections of the lesion were stained with BMP-specific pSmad 1, 5, 8 antibody and co-stained with CNS cell-specific markers. pSmad positive cells were then counted around the lesion. The remaining five rats (n=5/time point) had luciferase (blood spinal cord barrier permeability marker) injected through the jugular vein. Subsequently, spinal cords were collected and luciferase activity quantified around the lesion and in the cervical samples (controls) using a luminometer.

Results: After injury, a significant increase in the number of pSmad positive cells was observed when rhBMP-2 was implanted at the 30min, 24hrs and 7 day time-points ($p<0.05$). Co-staining revealed BMP-specific signaling activation in neurons, glial cells, macrophages and fibroblasts. Spinal cord permeability to luciferase was significantly increased at 30min, 24hrs and 7 days post-lesion ($p<0.05$). A significant linear regression was established between the extent of BSCB permeability and pSmad signaling ($r^2=0.66$, $p=0.000$).

Conclusions: Our results indicate that rhBMP-2 use around a spinal cord lesion elicits a robust signaling response within the spinal cord parenchyma. All CNS cell types and the invading fibroblasts are activated to the extent dependent on the integrity of the meningeal and BSCB barriers. Therefore, in the presence of a spinal cord injury and/or dural tear, rhBMP-2 diffuses intrathecally and activates a signaling cascade in all major CNS cell types, which may increase glial scarring and impact neurologic recovery.

INTRODUCTION

Injury to the spinal column can potentially lead to debilitating neurologic deficits including para- or quadriplegia. Even minor axial skeleton trauma can result in temporary disability during the vertebral column healing phase [1, 2]. In cases requiring surgical decompression of the spinal cord or spinal realignment, the vertebral column is usually fused to prevent further instability and insult to the cord. Historically, autologous iliac crest bone graft has served as the "gold standard" grafting material for spinal arthrodesis [3]. Despite the overall high fusion rates achieved with autologous bone, harvesting of the bone graft has been associated with additional morbidity, complications and pain at the donor site [4, 5].

Recently, bone morphogenetic proteins (BMPs), specifically BMP-2 and BMP-7, have been introduced as effective bone graft substitutes or extenders with healing rates approaching and/or exceeding those obtained with autologous bone [6-8]. Clinical use of BMPs obviates the need for iliac crest harvesting, thus eliminating donor site morbidity and complications [6]. Currently, recombinant human BMP-2 (rhBMP-2) packed inside an anterior interbody fusion device, is the only FDA-approved BMP that can be used for treatment of discogenic pain in the lower lumbar spine [9]. Favorable clinical results in this application, and improved healing of appendicular fractures stimulated increased use of rhBMP-2 "off-label" in the thoracic and cervical spine [10-13]. However, extended indications for rhBMP-2 have lead to an increasing number of complications reported in the literature, that include soft-tissue swelling, heterotopic bone formation and vertebral body osteolysis [14-17].

As the list of spinal disorders managed through rhBMP-2 induced arthrodesis continues to expand, multi-level trauma and associated vertebral column instability are becoming the next indication for fusion with rhBMP-2. At the same time little is known regarding the potential effects of the exogenous BMP-2 on spinal cord pathology. Research indicates that blood spinal cord barrier (BSCB) is disrupted in a 100% of traumatic spinal cord injury (SCI) cases [18]. The extent of BSCB permeability is then directly dependent on the severity of injury [19]. This phenomenon, coupled with a high rate of concomitant dural tears associated with high-energy trauma with SCI (up to 74%), potentially expose the spinal cord parenchyma to exogenous BMP-2 applied to the spinal column [20].

Indeed, BMP receptors are present in the adult spinal cord and endogenous BMPs (BMP-4 and BMP-7) are upregulated following SCI [21-23]. BMP signaling is mediated in part through Smad-dependent pathways. Bone morphogenetic protein binding to its receptors' leads to receptor-mediated phosphorylation of Smad1, Smad5 and Smad8 [24]. Once phosphorylated, Smads-1, 5 or 8 complex with Smad4, the co-Smad, and translocate to the nucleus where they regulate gene expression [25, 26]. Thus, detection of phosphorylated Smads is an indication of active BMP signaling. Indeed, a recent publication showed intrathecal upregulation of the phosphorylated Smad (pSmad) proteins following a demyelinating lesion of the spinal cord [22].

The above findings have led us to hypothesize that when there is concomitant SCI, exogenous BMP-2 used for spinal fusion will enter the spinal cord and activate a BMP

signaling response. Therefore, our objectives were: 1) to evaluate activation of the BMP-specific signaling cascade within the spinal cord following its application to the spine at different time-points following a SCI and 2) to examine if the extent of BMP signaling is dependent on the integrity of the BSCB and meningeal barriers.

MATERIALS AND METHODS

Animals:

A total of sixty-five adult female Sprague-Dawley rats (female, 250-275g) were used in this investigation (Charles River Laboratories). Animals were housed in the Laboratory of Animal Medicine and had unlimited access to food and drink throughout the experiments. All protocols were approved by our Institutional Animal Care and Use Committee (IACUC).

Surgical Procedures:

Spinal Cord Injury:

Following arrival at the housing facility rats were kept for 7 days pre-operatively to acclimate to the housing environment. On the day of the surgery general anesthesia was induced with a ketamine / xylazine cocktail injection (Ketamine 80mg/kg; Xylazine 10mg/kg; i.p.). Animals back was shaved and aseptically prepared using 70% alcohol swabs. Rats were placed prone on a heating pad and covered with a sterile drape with a cutout access to the posterior thoracic spine. Following longitudinal skin incision extending from T7-T11, the paraspinal musculature was dissected, exposing the T8-T10 laminae and transverse processes. Subsequently, partial laminectomy of the caudal part of the T9 and the cephalad aspect of T10 laminae was performed to allow direct access to the spinal cord. Dorsal hemisection of the spinal cord was performed under the microscope to a depth of 1.25mm. The depth of the spinal cord transection was established following pilot dissections of the rat spinal cord and corresponded to the thickness of the white matter columns from the dorsal surface. A single surgeon

performed all hemisections using microdissection scissors marked at 1.25mm from the tip. Following visual inspection of the lesion to verify complete posterior column transection, the paraspinal musculature was re-approximated using 6.0 Ethilon suture (Ethicon, Inc, Somerville, NJ). The cutaneous incision was closed with skin staples. Laminectomy only animals had identical procedures performed with the omission of the dorsal hemisection. Post-operative morbidity was managed using buprenorphine (0.03mg/kg s.q.), administered immediately after the surgery and on "as needed" basis thereafter. During the survival period, all rats were monitored twice daily, at which time the bladder was manually expressed until the recovered urinary volume dropped below 2ml for two consecutive times. Additionally, animals received prophylactic antibiotics (cefazolin sodium: 35mg/kg s.q.) once daily for 5 days post-op to control for post-operative infections.

Posterolateral Arthrodesis and Intrathecal rhBMP-2 penetration studies

For the experiments examining the penetrance of rhBMP-2 into spinal cord parenchyma, SCI was performed on thirty two (n=32) animals and subdivided into four follow-up groups with rhBMP-2 application occurring at: 30 minutes (n=8), 24 hours (n=8), 7 days (n=8) and 21 days (n=8) after induction of the injury. At these time points, a second procedure was performed that included surgical re-exploration of the T8-T10 laminae followed by application of either 43μg of rhBMP-2/per side (n=4) (Infuse™, Medtronic Spine and Biologics, Minneapolis, MD) or sterile water (n=4; control) on two absorbable collagen sponges (ACS) (20mm x 15mm x 3mm ACS / side). rhBMP-2 was added to the sponges 15 minutes prior to implantation at a concentration of 100μg/ml in sterile water

per manufacturer's recommendations. Eight additional rats (n=8) served as laminectomy only controls. In the laminectomy only animals ACS sponges with or without rhBMP-2 were implanted at the 30-minute post-lesion time-point (Fig. 1).

Tissue Collection

At 24 hours post ACS implantation, all animals were deeply anesthetized with ketamine / xylazine and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). The full-length spinal cord was then excised and post-fixed overnight at 4°C degrees. Tissue was cryoprotected in 30% sucrose in phosphate buffer (PBS) and maintained at 4°C for at least 72 hours or until sinking. Spinal cord sections surrounding the lesion (±5mm) were collected, embedded in OCT compound and stored at -80°C for later sectioning and immunohistochemical staining.

BSCB and Meningeal Barrier Permeability studies:

An additional group of twenty-five (n=25) rats were allocated to study the reformation of the BSCB and meningeal barriers after the dorsal hemisection SCI. Anesthesia, surgical procedures and SCI induction followed the protocol outlined above. Spinal cord permeability to luciferase was evaluated in animals at 30 minutes (n=5), 24 hours (n=5), 7 days (n=5), and 21 days (n=5) after dorsal hemisection and in control animals 30 minutes after laminectomy (n=5) (Fig. 2). At these time-points a second control surgery was performed consisting of re-exploration of the T8-10 spinal laminae to simulate ACS sponge implantation and re-create the local vascular bleeding. However, the incision was then closed using 6.0 Ethilon suture (Ethicon, Inc., Somerville, NJ) without ACS

implantation. The animal was re-positioned supine and the right jugular vein exposed. Luciferase (1mg/ml in Luciferase Storage Buffer; Promega) was diluted 1:1 with PBS/ 0.001% bovine serum albumin (BSA). The vein was cannulated with a 27ga needle and luciferase injected at a dose of 3.33μg/g body weight (Average=850μg per 250g rat) [27]. The animal remained sedated for 30 minutes after the injection, at which time it was euthanized with an overdose of the ketamine / xylazine cocktail (Ketamine 80mg/kg; Xylazine 10mg/kg; i.v.). A 3mm spinal cord segment centered over the lesion was excised, briefly rinsed in PBS, weighted and diluted 1:25 by weight in Cell Culture Lysis Reagent (Promega Corp., Madison, WI). A control 3mm spinal cord section was collected from the upper cervical region, weighed and diluted as above. The samples were then stored at -80°C for further processing.

Immunohistochemistry:

Spinal cord samples embedded in OCT were sectioned along the sagittal plane using a cryostat set at 20μm. Every one-in-twelve sections were collected and mounted on gelatin-coated glass slides for immunohistochemical labeling. Prior to staining, spinal cord sections were re-hydrated in PBS and blocked for one hour at room temperature in 5% goat serum with 0.03%Triton X-100 in PBS. Appropriate primary antibodies were then added and tissues incubated overnight at 4°C in the humidity chambers. To evaluate BMP-2 signaling within the spinal cord, a pan rabbit polyclonal anti-pSmad -1, -5, -8 antibody (1:150, Cell Signaling, Beverly, MA) was used. To further characterize the types of cells responsive to BMP, additional spinal cord sections were co-stained with anti-pSmad 1/5/8 antibody and the following cell specific markers: reactive astrocytes –

mouse monoclonal anti-GFAP (1:1000, Millipore, Billerica, MA), neurons – mouse monoclonal anti-β III tubulin (TUJ1/TU20, 1:500, Millipore, Billerica, MA), oligodendrocytes – mouse monoclonal anti-CC1 (1:100, EMD), macrophages/microglia – mouse monoclonal anti-ED-1 (1:175, Millipore, Billerica, MA), fibroblasts – mouse monoclonal anti-prolyl 4-hydroxylase β (1:750, Millipore, Billerica, MA). After washing three times in PBS, sections were incubated for 90 minutes with the appropriate secondary antibodies conjugated either to Alexa-488 or Alexa-568 (goat anti-rabbit and anti-mouse, respectively) (1:200, Molecular Probes, Eugene, OR). Slides were allowed to dry and were coverslipped with DAPI-containing mounting medium to label nuclei. To control for non-specific secondary antibody binding, a primary antibody omission control was included with each batch of slides stained.

Quantitative Analysis:

Quantification of the number of pSmad 1/5/8 positive cells was performed within the spinal white and gray matter. Spinal cord sections co-stained with the oligodendrocyte marker CC1 were used to differentiate between the white and gray matter. Prior to quantification, all tissues were coded to avoid sampling bias. Two fields within 1mm of the lesion epicenter were digitally photographed using an Olympus BX61 microscope with an attached CCD camera (Magnification 20X). Images were obtained from six spinal cord sections per animal that were separated by 240μm, generating a total of twelve (n=12) quantifiable fields. Images were captured using iVision-Mac™ Software (Bio-Vision Technologies, Exton, PA) followed by cell counting using NIH ImageJ Software (NIH, Bethesda, MD). To determine cell density, the number of cells positive

for pSmad 1/5/8 were counted within a constant predetermined region of interest (ROI) and divided by the total area of the ROI (area=0.105mm^2). Cell densities for the control and rhBMP-2 treated animals were tabulated and averaged at each time-point. The data are presented as means ± standard error of the mean (SEM) (Table 1).

Luciferase Assays:

Spinal cord sections (3mm around the lesion or control cervical samples) were homogenized using an electric homogenizer (Ultra-Turrax T8, Ika-Werke, Staufen, Germany) and centrifuged at 12,000rpm for 8 minutes to separate cellular debris. Supernatant was collected and duplicate 20µl aliquots of each sample loaded onto a 96-well plate. Luciferase activity was quantitated using the Luciferase Assay System (Promega Corp., Madison, WI) and read by a Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA). Luciferin was automatically injected into each well (100µl / well) followed by a 2 sec delay and 10 sec measurement of the luciferase activity.

Luminosity values obtained for the cervical samples served as internal controls for the lesioned area in each animal [27]. To determine the relative change in luciferase activity at the lesion site, the raw luminescence values for each lesioned segment were divided by the values for the respective cervical samples and reported as percent (%) change in luminescence.

Data and Statistical Analysis

Descriptive data are presented as means ± SEM. Intergroup differences between control and rhBMP-2 treated animals were compared using a Student's t-test at each follow-up point. Within group comparisons (across the time-points) were evaluated using an analysis of variance (ANOVA) followed by either a Tukey's Honestly Significant Difference (HSD) or Fisher's Least Significant Difference (LSD) tests as post hoc comparisons. Linear regression relationship was established by calculation of an R^2 coefficient. All statistical computations were performed using the SPSS 16.0 software (SPSS, Inc., Chicago, IL) and difference at $p<0.05$ considered significant.

RESULTS

BMP signaling response

To evaluate whether rhBMP-2, implanted in the ACS on the spinal column, was able to initiate intrathecal signaling, we examined staining for the BMP-activated phosphoSmads -1, -5 and -8 in spinal cords from animals after either dorsal hemisection or laminectomy only.

Overall BMP activity and cellular co-localization

We observed BMP signaling through detection of pSmad in spinal cord from all animals, whether implanted with rhBMP-2 or control ACS, indicating a basal level of pSmad activity after SCI. In the animals implanted with control ACS this amounted to a minor upregulation of pSmad 1/5/8 staining at the later time-points following SCI (7dpl and 21dpl) that was not statistically significant (ANOVA: $F=0.266$, $p=0.894$). In contrast, we observed a significant increase in the density of the pSmad positive cells following rhBMP-2 implantation at 24 hours and 7 days after SCI relative to control ACS surgery ($p=0.026$ and $p=0.003$, respectively) (Fig. 3). When rhBMP-2 ACS were implanted 30 minutes or 21 days after SCI we observed a trend for increased pSmad signaling; however, these differences did not reach statistical significance ($p=0.098$ and $p=0.068$, respectively). These results, combined with the statistically greater values from the 24 hour and 7 dpl groups, highlight a strong, persistent trend for the increased intrathecal BMP signaling that follows its implantation near the spinal cord at any time point. pSmad immunoreactivity was co-localized with DAPI, showing that its activity was exclusively nuclear. Thus, BMPs were able to penetrate spinal cord parenchyma, bind to cell surface

receptors and initiate an intracellular signaling response leading to pSmad translocation to the nucleus.

Additional serial sections through the lesioned spinal cord were co-stained with pSmad 1/5/8 and cell-specific markers for neurons, oligodendrocytes, astrocytes as well macrophages/microglia and meningeal fibroblasts. Immunofluorescent analysis of these sections revealed robust nuclear pSmad staining in each of the above cell types (Fig. 4).

Two laminectomy-only groups (±rhBMP-2) were included in the study to control for the surgical resection of the spinal lamina and potential rhBMP-2 infiltration through the intact meninges. All animals survived the surgery and exhibited no signs of locomotor deficiency post-operatively. Extra care was taken to avoid iatrogenic dural tears during the laminectomy and there were no cases of cerebrospinal fluid (CSF) leakage. In animals that had control-ACS implanted after laminectomy there was detectable endogenous BMP signaling through pSmad staining in all major CNS resident cell types. Implantation of the rhBMP-2 impregnated collagen sponges over the laminectomy defect did not contribute to a significant increase in pSmad 1/5/8 activity ($p=0.060$). We did, however, observe a strong trend for a greater number of pSmad 1/5/8 positive cells in both the white and gray matter of the rhBMP-2 treated animals indicating potential intrathecal BMP-2 penetration through intact spinal meninges. Additional serial sections co-stained with pSmad and cell specific markers indicated BMP signaling in neurons, oligodendrocytes and astrocytes.

White Matter

To better delineate the effects of rhBMP-2 on intrathecal pSmad 1/5/8 signaling following SCI, we performed separate cell counts in the white and gray matter around the lesion. We found the greatest differences between the control and rhBMP-2 treated animals in the white matter (Fig. 5a). Following dorsal hemisection, implantation of rhBMP-2 on an ACS resulted in a significant upregulation of pSmad immunoreactivity in the white matter adjacent to the lesion ($p<0.05$). These finding were observed when rhBMP-2 was implanted 30 min, 24 hrs or 7 dpl ($p=0.017$, $p=0.005$, $p=0.013$, respectively). In the experimental group, the greatest overall increase in the number and density of the pSmad 1/5/8 positive cells was observed at 7dpl (225% increase). Immunofluorescent analysis suggested that this could be attributed to a pronounced rise in pSmad 1/5/8 immunoreactivity within the meningeal fibroblasts invading the glial scar and the activated microglia/macrophages surrounding the lesion (Fig. 6). By 21 dpl, rhBMP-2 implantation to the spine did not contribute to a significant increase in pSmad 1/5/8 immunolabeling ($p=0.083$) and approximated cell density in the laminectomy only group.

Gray Matter

In spinal gray matter, the endogenous pSmad immunoreactivity was higher than in the white matter of the same sections. Nevertheless, intergroup differences between the rhBMP-2 treated animals and control rats adhered to the same trends at all time-points following a SCI. The greatest increase in the density of pSmad positive cells around the lesion was observed at 7 dpl ($p=0.005$) (Fig. 5b). At the earlier time-point (30min and 24

hrs post-lesion) rhBMP-2 delivery to the spine contributed to a marginal increase in pSmad immunoreactivity within the gray matter that failed to reach statistical significance (p=0.343 and p=0.165, respectively). Similarly to the data obtained within the white matter, there was a reduction in the number of pSmad positive cells when rhBMP-2 was implanted 21 days after the SCI relative to the 7dpl time-point.

Blood Spinal Cord and Meningeal Barrier Permeability

To investigate whether intrathecal penetration of the exogenous rhBMP-2 is related to the integrity of BSCB and meningeal barriers we evaluated barrier permeability to firefly luciferase. Following an IV injection, we observed no differences in luciferase activity within the spinal cord samples obtained from the laminectomy site and the control cervical samples. This finding reinforced our intra-operative observation that lamina excision alone did not affect BSCB permeability and maintained dural integrity. In contrast, immediately following dorsal hemisection, spinal cord permeability to luciferase increased more than twofold (ANOVA: F=8.34; p=0.014) (Fig. 7). Lesion site luminosity peaked between 24 hours and 7 days after the injury, with values rising by over 350% compared to the cervical controls (p=0.000 and p=0.000, respectively). By 21 days, however, BSCB and meningeal barriers appear to reform with a dramatic drop in permeability approximating that of the uninjured control tissues (p=0.461). To further assess the relationship between intrathecal BMP-2 signaling changes and meningeal healing, a linear regression analysis was performed. We established a highly significant predictive relationship between the state of BSBC/meningeal disruption and the extent of

intrathecal pSmad signaling following rhBMP-2 application to the spine (R^2=0.662; p=0.00006) (Fig. 8).

DISCUSSION

As clinical experience and availability of rhBMP-2 continue to expand, the list of spinal disorders managed with this bone graft substitute/extender is also evolving and now includes pathology of the thoracic and cervical spine. More recently, traumatic segmental instability with or without concomitant spinal cord pathology has been addressed using ”off-label” application of rhBMP-2 posterolaterally (personal communication at the 22nd Annual Meeting of the North American Spine Society). At the present moment there are no published reports of any neurologic complications directly triggered by rhBMP-2; however, profound soft tissue swelling and inflammation have been observed in the cervical region [15]. Furthermore, within the spine surgeon community, current concerns that exist as to the use of rhBMP-2 near the spinal cord are related to heterotopic (HO) bone formation into the spinal canal and the associated mechanical compression of the cord [16]. Surprisingly, little attention is paid to the potential direct effects of the exogenous BMPs on the cells comprising the spinal cord.

As a first step in this direction, we performed a comprehensive evaluation of rhBMP-2 induced intrathecal signaling in the presence of a dorsal hemisection SCI. Clinical cases of high-energy spinal trauma frequently involve dural and spinal cord lacerations [20]. Therefore, we chose this model of SCI to simulate the worst-case scenario for exposing the spinal cord parenchyma to the exogenous protein. Early implantation of rhBMP-2 (up to 7dpl) resulted in a significant increase in the number of pSmad positive cells in comparison to implantation of the water-containing ACS, showing that rhBMP-2 was indeed able to penetrate the spinal cord parenchyma and elicit a biological response. To

our knowledge this is a first study documenting a profound intrathecal signaling response secondary to rhBMP-2 use in posterolateral arthrodesis in the presence of an SCI.

Our results indicate that all major CNS-resident cell types are responsive to BMP signaling. Nuclear pSmad staining was observed in cells co-labeled with specific markers for neurons, astrocytes and oligodendrocytes. Other cell types that were positive for pSmad include macrophages and activated microglia, as well as invading fibroblasts. Increased BMP signaling in these cells could have a significant impact on the inflammatory response to injury and subsequent secondary cell death. It could also alter the composition of the glial scar that forms after injury. All these responses could affect neurologic recovery, particularly after an incomplete injury.

Tissue gliosis, ensuing after injury to the CNS, is one of the major impediments to axonal regeneration and functional recovery [28]. Reactive astrocytes, that are the most populous cell type within the glial scar, produce a number of chondroitin sulfate proteoglycans (CSPGs), which are highly inhibitory to axonal growth [29]. Previous studies have shown that BMP-4 and BMP-7 stimulate CSPG expression in cultured astrocytes [22]. *In vivo*, direct injection of BMP-4 and BMP-7 into the dorsal columns triggered a localized expression of CSPGs around the site [22]. As BMP-4 and BMP-2 share greater than 90% homology in the amino acid sequence and mature peptide structure [30], it is plausible to suspect that rhBMP-2 could also trigger CSPG expression in the spinal cord.

Other findings have shown that BMP-2 exerts a direct inhibitory effect on neurite outgrowth in cultured cerebellar neurons [31]. *In vivo*, inhibition of endogenous BMP signaling via a local injection of a BMP antagonist, noggin, has been shown to improve axonal regeneration and functional recovery following a SCI [32]. Thus, these data raise concerns of whether exogenous rhBMP-2 could inhibit axonal regeneration through a direct neuronal interaction.

Macrophages and microglia are known modulators of the inflammatory response and associated secondary cell death [33]. The direct effects of BMPs on macrophages have recently been studied in culture using BMP-6 [34]. The authors report that addition of BMP-6 to macrophage culture resulted in increased expression of pro-inflammatory inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF-α). Following SCI, both of these molecules have been associated with increased secondary injury and cell death [33]. Therefore, rhBMP-2 may also induce a similar response in macrophages invading the lesioned area following SCI.

With respect to the number of cells positive for pSmad, we observed greater increases in rhBMP-2 mediated signaling in the white matter. One plausible explanation could be the physical proximity of the white matter columns to the periphery of the spinal cord, and hence to the rhBMP-2 soaked sponges on the spine. Conversely, there was a stronger background pSmad signaling in the gray matter of the uninjured cord, a region that is predominantly neuronal. Therefore, the post-injury activation of BMP signaling in astrocytes and oligodendrocytes, which make up the dorsal column cell population, may

have contributed to an immediate change in white matter pSmad counts. Furthermore, the most pronounced differences between pSmad activation in grey and white matter were observed at 7dpl. This finding correlated with the prominent increase of pSmad immunoreactivity in the meningeal fibroblasts invading the glial scar and the activated microglia/macrophages surrounding the lesion. Qualitatively, these cells appeared to localize within the white matter surrounding the lesion. Our results are in concordance with the work by Batchelor et al [35] who demonstrated that SCI generates a greater inflammatory response in the white matter with peak numbers of macrophages and microglia recruited around 7dpl. Thus, the largest increase in pSmad labeling correlates with the time when the highly BMP-responsive cell types infiltrate the white matter surrounding the lesion. Although the exact effects of rhBMP-2 on macrophages and microglia are unknown, previous *in vitro* work with BMP-6 raises concerns for a possible increased inflammatory response [34].

As a secondary objective of this study we correlated the extent of BMP-2 induced pSmad activation with the BSCB and meningeal barrier permeability. In agreement with previous work, our results indicate an immediate disruption of the BSCB and meningeal barriers, which peaked at 7dpl [27]. We established a highly significant correlation and a linear regression relationship between the protective barrier damage and intrathecal pSmad signaling. Furthermore, as the BSCB appeared to reform at 21dpl, pSmad immunoreactivity in the rhBMP-2 treated animals returned to pre-injury levels. Of note, however, is a persistent trend for increased pSmad signaling in both the laminectomy only and 21dpl SCI groups treated with rhBMP-2. In both groups there was a respective

26% and 27% higher total density of pSmad positive cells, than in animals implanted with the control ACS. These differences, while not statistically significant, suggest that rhBMP-2 may penetrate through the intact meninges. This concept warrants further investigation as it has significant clinical implications.

In conclusion, our data clearly indicate that rhBMP-2 triggers a direct intrathecal signaling response when used in animals with a spinal cord injury. Invading inflammatory modulators and all CNS cell types are affected with the magnitude of cellular activation dependent on the integrity of the meningeal and blood spinal cord barrier. Based on the results of this project, additional animal studies are ongoing that will define the morphologic changes within the spinal cord triggered by rhBMP-2, and assess whether these changes affect long-term neurologic recovery. Our current findings warrant serious consideration for the pre-operative planning and clinical use of rhBMP-2 in patients with spinal cord pathology or significant dural deficiencies.

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Tables and Figures

Table 1: Intrathecal density of pSmad1/5/8 positive cells				
	ACS Implantation Time Point After SCI			
	30min	**24 hours**	**7 days**	**21 days**
Overall Density (Cells/mm^2)				
rhBMP-2	2171±47	2676±212	3378±280	2448±194
Control	1824±212	1812±206	1978±88	1931±182
White Matter				
rhBMP-2	949±26	1135±112	1374±203	800±81
Control	712±67	648±27	613±84	578±97
Gray Matter				
rhBMP-2	1300±47	1540±201	2005±106	1648±124
Control	1111±177	1165±155	1374±41	1353±94

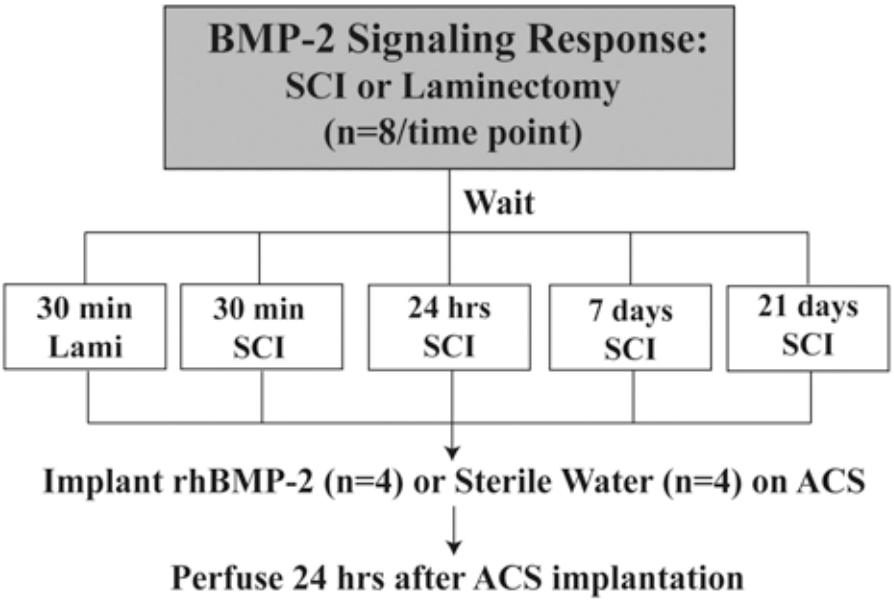

Figure 1: Schematic diagram outlining treatment and surgical groups used for the study of rhBMP-2 induced intrathecal signaling. At the respective time-points either rhBMP-2 or sterile water containing ACS sponges were implanted over the T8-T10 spinal lamina and left in place for 24 hours.

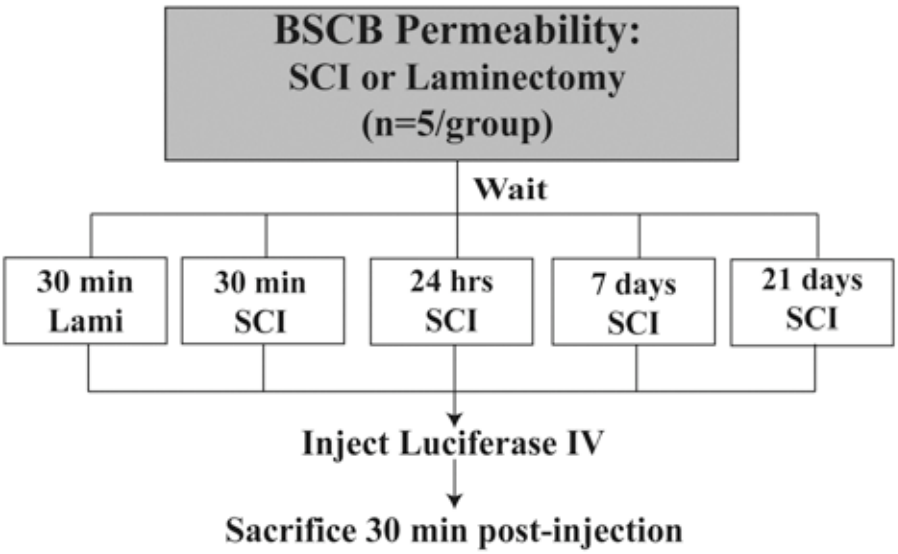

Figure 2: Schematic representation of the surgical groups and follow-up time-points used for the study of BSCB and meningeal barrier permeability. Luciferase was injected IV at the same time-points as outlined in Figure 1 and animals sacrificed 30 minutes later. This allowed for a direct comparison between the state of the protective barrier and the extent of rhBMP-2 induced intrathecal signaling.

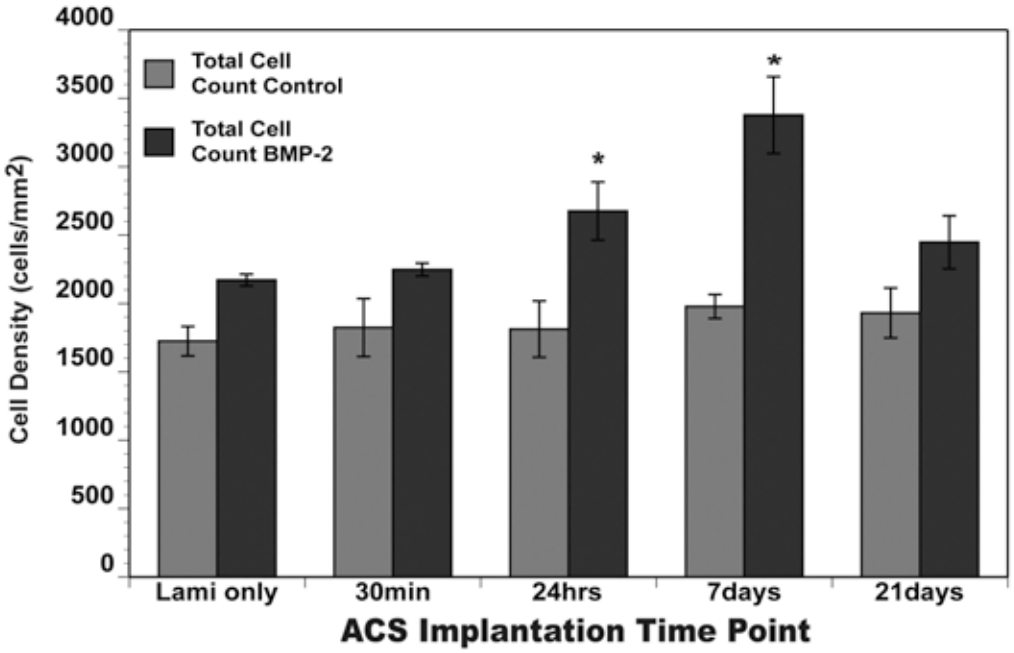

Figure 3: Total intrathecal pSmad immunoreactivity. This bar chart represents the overall density of the pSmad positive cells within 1mm circumference around the lesion. In the laminectomy groups there were marginal differences between rhBMP-2 treated and control animals (t-test; p=0.06). In the SCI groups a significant increase in the density of pSmad positive cells was recorded when rhBMP-2 was implanted 24 hours post-lesion (t-test; p=0.026) and 7 dpl (t-test; p=0.003). At 21dpl pSmad counts were not statistically different between control and rhBMP-2 soaked sponges (t-test; p=0.068). Within-group analysis of the rhBMP-2 group indicated the cell density increase observed at 7dpl to be significantly higher than at all other time-points (ANOVA: F=5.66, p=0.006). All data represent mean ± SEM.

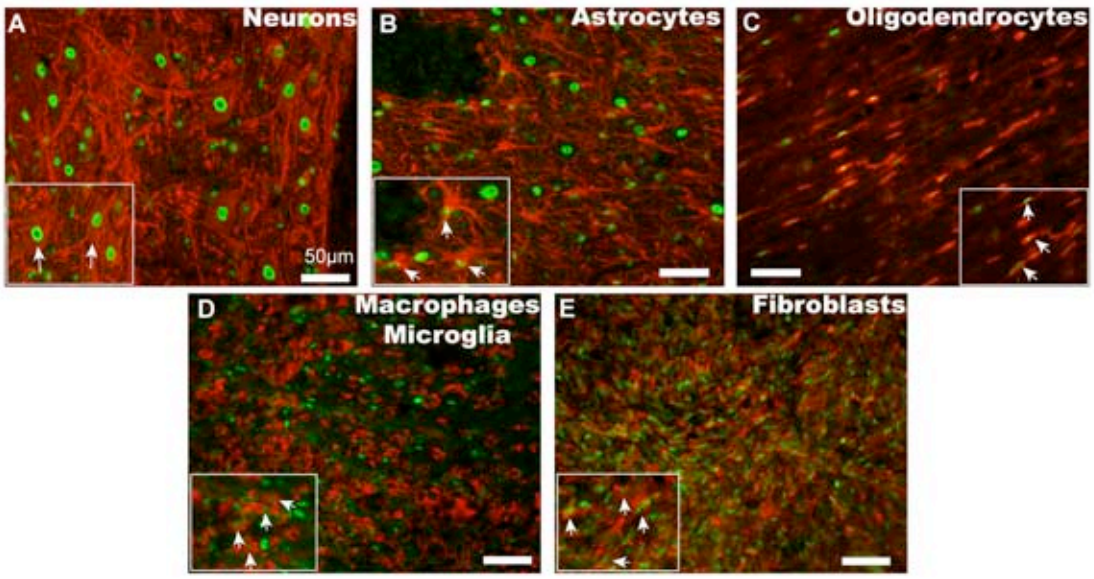

Figure 4: Longitudinal sagittal sections through the lesioned spinal cord. Robust nuclear pSmad labeling (green) was observed in all CNS-resident cell types (red). Specific pSmad co-localization (arrows) is shown in sections co-stained with cell markers for (A) neurons (Tu20), (B) astrocytes (GFAP), (C) oligodendrocytes (CC1), (D) macrophages / microglia (ED-1), and (E) fibroblasts (prolyl-4-hydroxylase β). Scale bar 50μm. Magnification 20X.

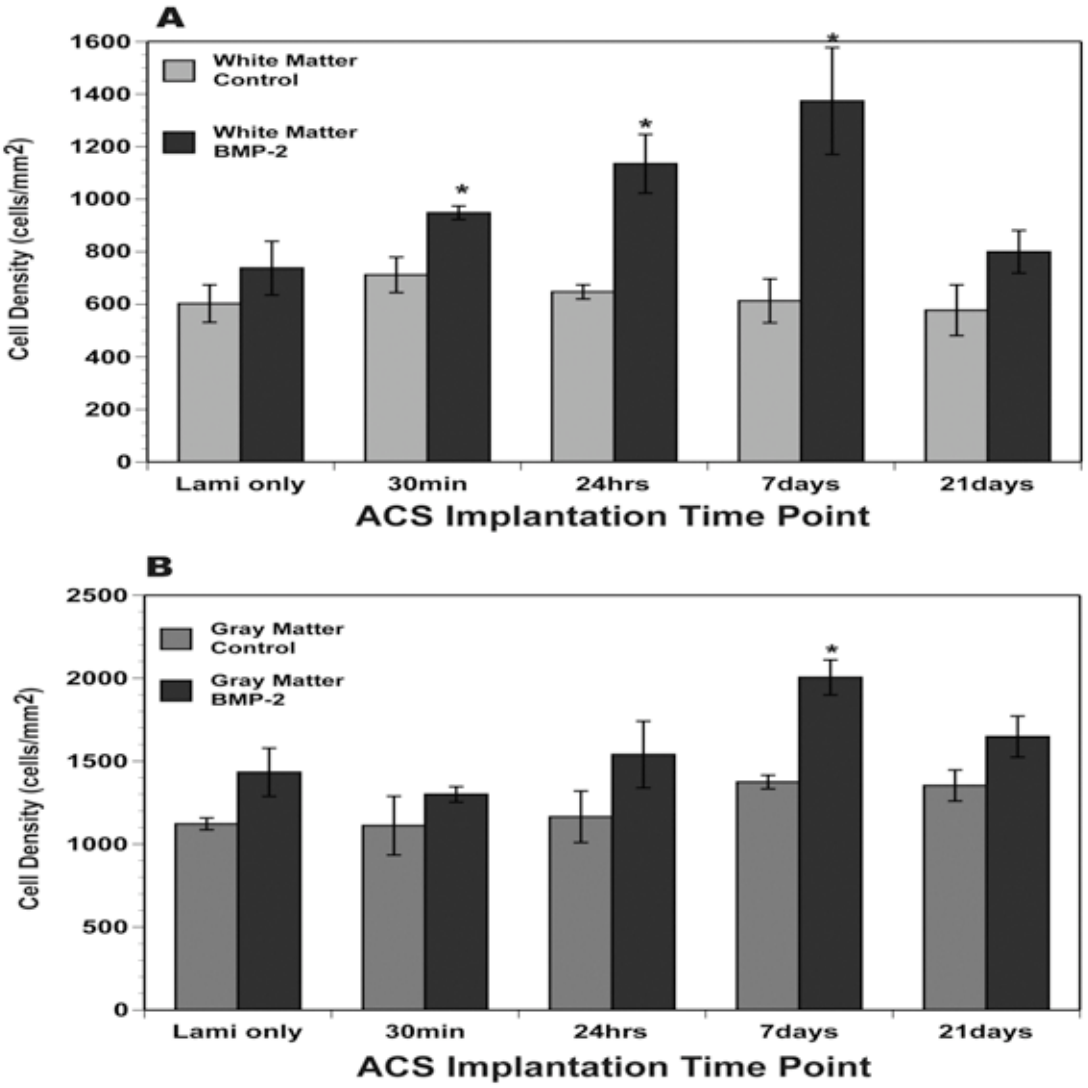

Figure 5: Quantification of the pSmad positive cells in the spinal cord white and gray matter. rhBMP-2 implantation to the spine triggered a greater signaling response in the cells of the white matter compared to the cells in the gray matter. In the white matter (A) significant differences in the density of pSmad positive cells were recorded at 30 minutes, 24 hours, and 7 days after injury (t-test: $p=0.017$, $p=0.005$, $p=0.013$, respectively). In laminectomy only (t-test; $p=0.177$) and 21dpl SCI (t-test; $p=0.083$) groups there were no statistical differences between the control and rhBMP-2 treated animals. Analysis of the gray matter (B) indicated greater endogenous BMP signaling, which was predominantly nuclear. Only following rhBMP-2 implantation at 7dpl was there a significant increase in the number of pSmad positive cells (t-test; $p=0.005$). All data represent mean ± SEM.

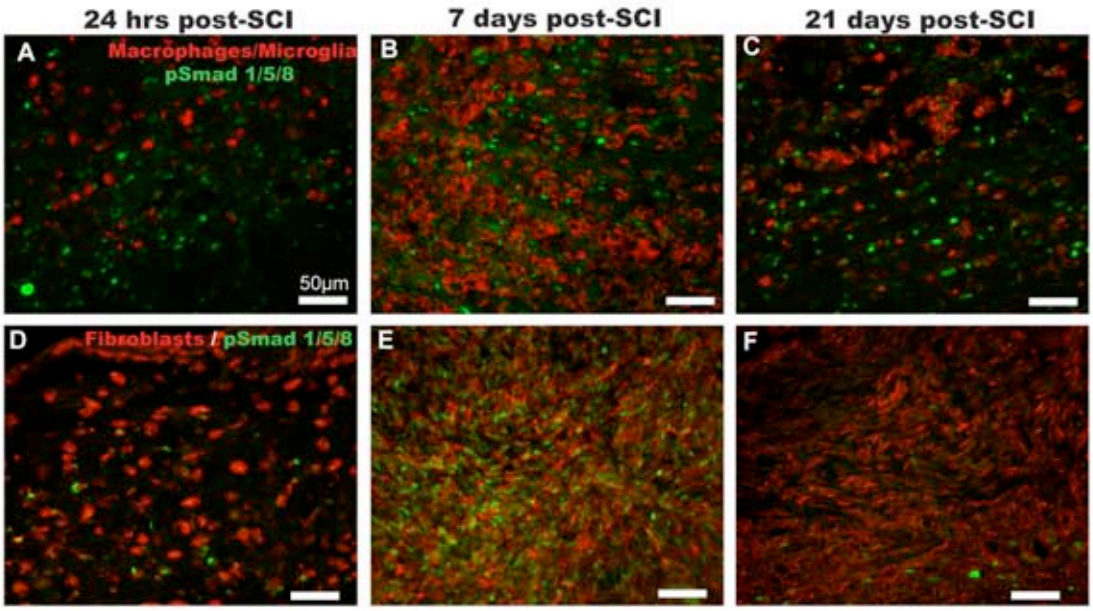

Figure 6: Immunohistochemical staining of the longitudinal spinal cord sections near the lesion with pSmad (green) and cell markers for (A-C) macrophages/microglia (red) and (D-E) fibroblasts (red). A significant rise in pSmad immunoreactivity in the 7dpl group treated with rhBMP-2 was attributed to the activation of microglia and the invasion of macrophages and fibroblasts at that time point. Compared to 24hrs (A & D) and 21 days after injury (C & F), the 7dpl spinal cord sections (B & E) revealed a significant increase of the pSmad positive macrophages (B) and fibroblasts (E). Scale bar 50µm. Magnification 20X.

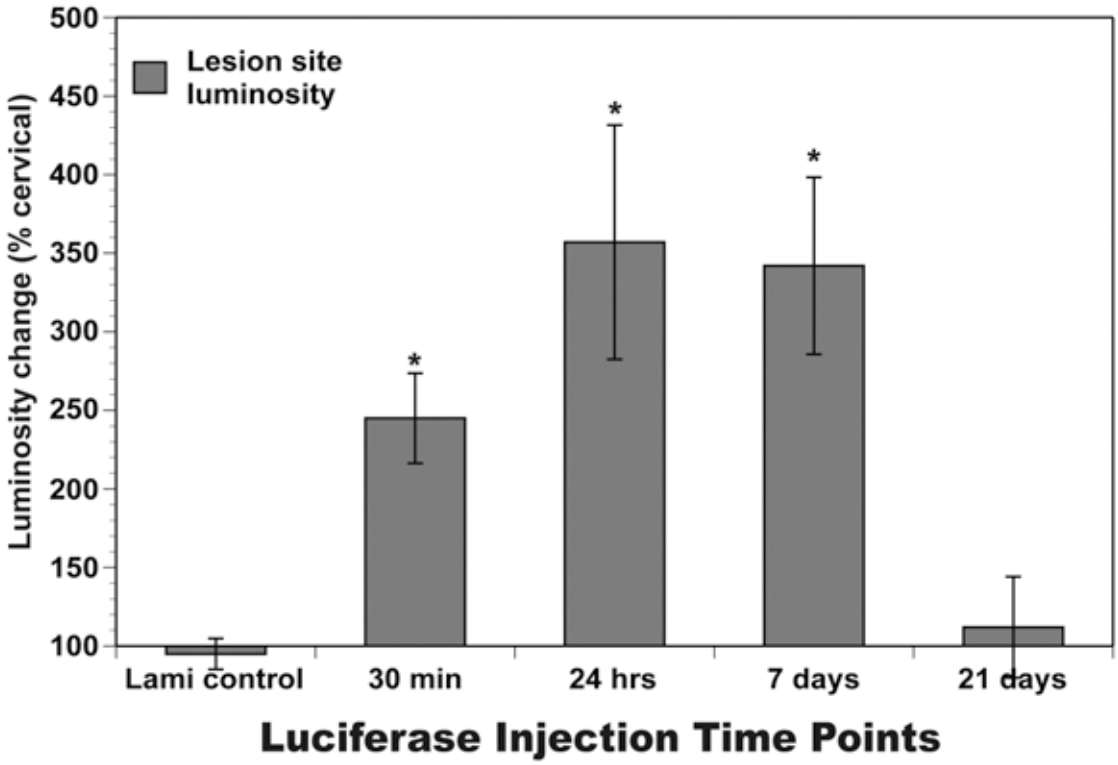

Figure 7: Time-course of spinal cord permeability to luciferase. Luciferase activity recorded in a 3mm section around the lesion was normalized to luminosity of a cervical tissue sample obtained from the same animal. In animals undergoing laminectomy, spinal cord samples from the cervical and thoracic region had approximately identical luminosity values. Following dorsal hemisection SCI, there was a significant increase in luciferase activity near the lesion epicenter that peaked between 24 hours and 7 days post-lesion indicating that luciferase entered the spinal cord parenchyma at these time points (ANOVA: $F=8.34$, $p=0.000$). All data represent mean ± SEM.

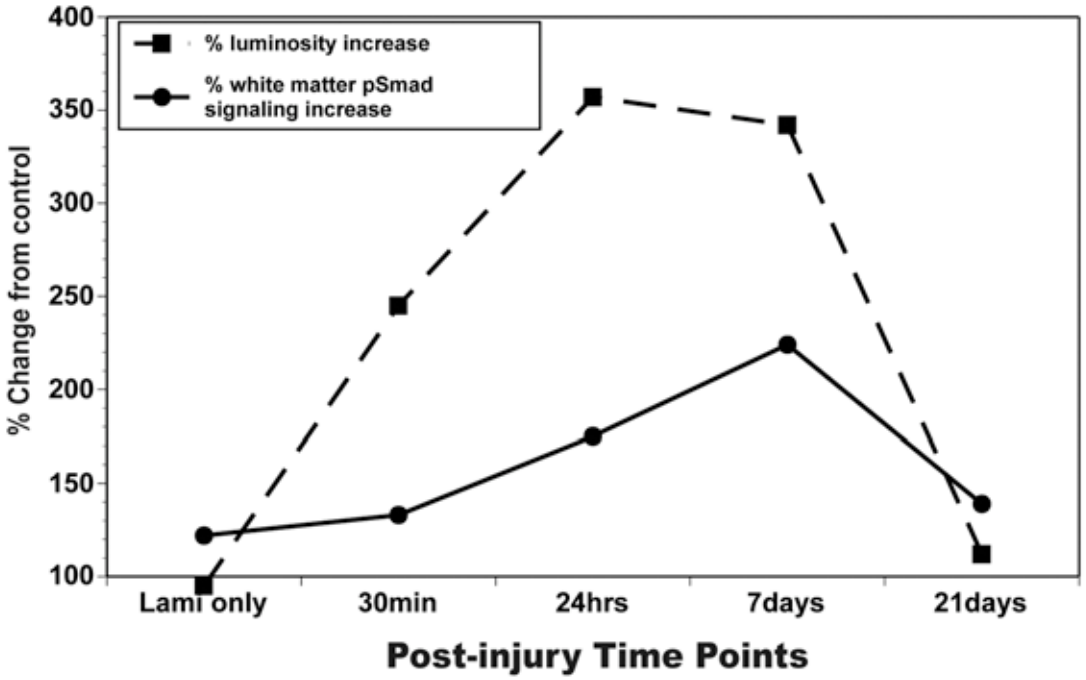

Figure 8: Time-course of BSCB leakage to luciferase and pSmad signaling increase in the white matter. This time course line chart represents % change in luciferase activity around the lesion relative to the control cervical samples and % increase in the white matter pSmad immunoreactivity in rhBMP-2 treated animals compared to that of control treated animals . The density of pSmad positive cells increased in direct correlation with the breakdown of the BSCB and meningeal barriers. According to the linear regression statistic, the state of BSCB permeability was highly predictive of the extent of intrathecal rhBMP-2 penetration and signaling (R^2=0.662; p=0.00006).

CHAPTER 3

Alterations in the recovery from spinal cord injury when using recombinant human Bone Morphogenetic Protein-2 for posterolateral arthrodesis.

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Running Head: Acute and Long-Term Effects of rhBMP-2 on Spinal Cord Pathology

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Abstract

Recombinant human bone morphogenetic protein 2 (rhBMP-2) is a bone graft substitute that is currently used “off label” to treat spinal instability following trauma. However little is known of the direct effects of rhBMP-2 on the injured spinal cord. We have investigated the acute and long-term morphological and functional effects of using rhBMP-2 posterolaterally at the level of a dorsal hemisection spinal cord injury (SCI) in rats. One week post lesion, rhBMP-2 treatment significantly elevated the expression of ED1, GFAP and vimentin, indicating increased numbers of macrophages/ microglia, reactive astrocytes and invading fibroblasts around the lesion relative to control animals that underwent SCI without rhBMP-2 treatment. Additionally there was increased staining for chondroitin sulfate proteoglycans. Similar morphologic differences between the groups persisted at 6 weeks after injury. Functionally, in the rhBMP-2 treated animals at one week post lesion we found deficits in locomotor function as assessed by BBB scores and footprint analysis relative to control animals. By 6 weeks post lesion rhBMP-2 treated rats had equivalent BBB scores as the control animals but retained significantly greater paw angle changes than the vehicle control group. Our findings indicate that clinical use of rhBMP-2 in the vicinity of a SCI may have detrimental effects on neurologic recovery, particularly, in cases of incomplete spinal cord injury.

INTRODUCTION

Bone morphogenetic protein-2 has emerged as a potent osteoinductive agent that is currently FDA-approved for lumbar interbody fusion and long-bone trauma repair (FDA, 2002). Since the 2002 approval for interbody fusion in the anterior lumbar spine, rhBMP-2 in combination with a type-I collagen sponge, has gained in popularity among surgeons as an effective bone graft substitute (Valentin-Opran et al., 2002). In addition to high successful fusion rates, the use of rhBMP-2 obviates the need for autologous bone graft harvesting and eliminates morbidity and complications associated with the procedure (Mummaneni et al., 2004; Villavicencio et al., 2005). As clinical experience with rhBMP-2 continues to grow, many surgeons have begun "off-label" use of the product in all spinal regions (Boden et al., 2002; Baskin et al., 2003; Glassman et al., 2007b; Glassman et al., 2007a). In consequence, new complications emerged that were not observed during the initial studies. In the cervical region extreme soft-tissue swelling has been reported, whereas heterotopic bone formation and vertebral body osteolysis have been documented in the thoracic and lumbar spine (Shields et al., 2006; Smucker et al., 2006; Lewandrowski et al., 2007; Wong et al., 2008).

Vertebral column trauma is frequently associated with neurologic deficits of varying severity (Sekhon and Fehlings, 2001). Surgical management of multi-level spinal instability through arthrodesis with rhBMP-2 is becoming the next indication for fusion using the protein. However, limited data exist as to the direct effects of rhBMP-2 on the course of post-traumatic spinal cord pathology. Pre-clinical studies have demonstrated disruption of the blood spinal cord barrier (BSCB) in a 100% of cases with traumatic spinal cord injury (SCI) (Maikos and Shreiber, 2007). In addition, the etiology of cases of

multi-level spinal trauma often involves high-energy forces, which in turn have been associated with a dural tear rate of up to 74% (Bellabarba et al., 2006). Disruption of the BSCB and/or meningeal barriers potentially provides direct access to the exposed spinal cord parenchyma for the exogenous protein applied to the spine .

We have previously shown that rhBMP-2 can penetrate intrathecally when used for spinal arthrodesis near a dorsal hemisection SCI lesion (Dmitriev et al., 2009). The extent of BMP signaling was dependent on the integrity of the BSCB. Collagen sponges with rhBMP-2 implanted in the vicinity of the lesion at 30 minutes, 24 hours or 7 days post-SCI, triggered a profound increase in direct intrathecal BMP signaling in all cell types in the spinal cord (Dmitriev et al., 2009). However, nothing is known of the long-term morphologic or functional effects of rhBMP-2 on the spinal cord after injury.

Therefore, the current study was undertaken to 1) evaluate the acute effects of the posterolateral application of rhBMP-2 on the morphology of a spinal cord lesion and 2) to characterize these changes and determine whether there were functional alterations in a long term setting of post-SCI arthrodesis with rhBMP-2.

MATERIALS AND METHODS

Animals:

A total of fifty-four adult female Sprague-Dawley rats (female, 250-275g) were used in this investigation (Charles River Laboratories). Animals were housed in the Laboratory of Animal Medicine in a reverse light-dark cycle room with *ad libum* access to food and water throughout the experiments. All protocols were approved by our Institutional Animal Care and Use Committee (IACUC).

Randomization Schedule and Treatment Groups:

During the acclimation period a treatment randomization schedule was created with all animals assigned to one of the two groups: 1) Vehicle Control (n=24) or 2) rhBMP-2 (n=24). Within each group, animals were further subdivided according to the post-operative follow up period: acute: 1 week (n=8) or chronic: 6 weeks (n=16). For the acute follow up, an additional control group was included with animals implanted with rhAlbumin (n=4), to account for any non-specific cross-species immune response elicited by a human protein in a rat. Furthermore, two additional rats underwent laminectomy without SCI to control for any morphological or functional changes that may result from bone resection alone.

In the experimental treatment group 43μg rhBMP-2 was delivered to each side of the spine on an absorbable collagen sponge (ACS) (20mm x 15mm x 3mm ACS / side) (Infuse™, Medtronic Spine and Biologics, Minneapolis, MD). rhBMP-2 was added to the sponges 15 minutes prior to implantation at a concentration of 100μg/ml in sterile water per manufacturer's recommendations. Control animals received ACS sponges soaked in

an equal volume of sterile water. Animals in the albumin group (acute follow-up only) were implanted with 43μg of rhAlbumin (per side) dripped onto the same size ACS sponges.

Surgical Procedures:

Spinal Cord Injury:

Following delivery to the animal facility, rats were allowed to acclimate to the housing environment for 7 days. On the day of the surgery general anesthesia was induced with a ketamine / xylazine cocktail injection (Ketamine 80mg/kg; Xylazine 10mg/kg; i.p.). Animals back was shaved and aseptically prepared with 70% alcohol swabs. Rats were placed prone on a heating pad and covered with a sterile drape with cut out access to the posterior thoracic spine. The skin was incised midsagitally over the spinous processes of T7-T11. Paraspinal musculature was carefully dissected, exposing the T8-T10 laminae and transverse processes. Subsequently, partial laminectomy of the caudal part of the T9 and the cephalad aspect of T10 laminae was performed to allow direct access to the spinal cord. Dorsal hemisection of the spinal cord was performed under the microscope to a depth of 1.25mm. The depth of the spinal cord transection was established as described previously (Dmitriev et al., 2009). A single surgeon performed all hemisections using microdissection scissors that were marked at 1.25mm from the tip. Using the microscope the lesion was then visually inspected to verify complete posterior column transection. Upon verification, the wound was covered with a sterile saline-soaked gauze and the animals were left undisturbed for 30 minutes.

During the waiting period, ACS sponges were prepared with rhBMP-2, sterile water or rhAlbumin according to the randomization schedule described above. At 30 minutes post-lesion, the wound was gently re-explored and the sponges implanted bilaterally over the transverse processes of T8-T10. Following implantation, the paraspinal musculature was re-approximated using 6.0 Ethilon suture (Ethicon, Inc, Somerville, NJ) and the cutaneous incision was closed with skin staples. Laminectomy only animals had identical procedures performed with the omission of the dorsal hemisection. Post-operative morbidity was managed using buprenorphine (0.03mg/kg s.q.), administered immediately after the surgery and on "as needed" basis thereafter. During the survival period, all rats were monitored twice daily, at which time the bladder was manually expressed until the recovered urinary volume dropped below 2ml for two consecutive times. Additionally, animals received prophylactic antibiotics (cefazolin sodium: 35mg/kg s.q.) once daily for 5 days post-op to control for post-operative infections. Rats developing late-onset autophagia were managed with acetaminophen p.o. and local application of antibiotic ointment either once or twice daily (depending on the severity).

Two animals were lost intra-operatively secondary to anesthesia complications. They were replaced with additional animals. There were four cases of post-operative urinary tract infections that were successfully managed with antibiotics. In the control group, 35% of animals developed autophagia, which is a known manifestation of post-SCI neuropathic pain in rats (need reference). However, among animals treated with rhBMP-2, 56% developed the condition post-operatively. All but one were successfully managed with acetaminophen and did not require premature euthanasia.

Cortical Injection Surgery and Anterograde Labeling

Eight animals from the 6 week control and rhBMP-2 groups (n=8/group) were selected to undergo anterograde labeling of the motor cortex with a fluorescent dye. Ten days before sacrifice (32 dpl), cortical injection surgery was performed and a total of 12μl of the 5% tetramethylrhodamine biotinylated dextran (mini-ruby, Molecular Probes) was injected into 6 stereotaxic coordinates (2μl of mini-ruby per site). This fluorescent marker is hydrophilic and biologically inert. Upon injection into the cortex, the dye is incorporated in the motor neurons and undergoes anterograde transport within the cytoplasm to produce fluorescent labeling of the descending axons within the spinal cord. In preparation for the surgery animals were anesthetized with a ketamine /xylazine cocktail (Ketamine 80mg/kg; Xylazine 10mg/kg; i.p.)., skull shaved and aseptically prepared with alcohol swabs. The animal was then placed on a heating pad and the head secured in the stereotaxic frame. A longitudinal skin incision overlying the sagittal suture was performed, exposing the skull. Using a high-speed electric burr and a 1.0mm circular drill-bit (Midas-Rex, Medtronic, Memphis, TN) two parallel troughs were made in the skull starting at ±1.5mm to the left and right of bregma at the level of the coronal suture and extending 3.0mm caudally along the sagittal suture. Sterile saline was dripped on the skull to ovoid overheating and care was taken not to violate the underlying dura. The axonal tracer (mini-ruby) was then injected into the brain at six previously validated stereotaxic coordinates (2μl per site) for the rat primary motor cortex (from bregma: -0.11mm AP and ±1.60mm ML; -1.33mm AP and ±1.50mm ML; -2.85mm AP and

±1.40mm ML) . The depth of the injections was set at 1.2mm from the dorsal surface of the dura. Using a 27ga needle attached to a 20µl hamilton syringe the dye was injected at a rate of 0.5µl per minute. Following injections, the skin was subqutaneously closed with a 6.0 ethilon suture.

Functional Testing

Open Field Locomotion

Open field ambulation and gross motor recovery were assessed according to the Basso, Beattie and Bresnahan (BBB) scoring system (Basso et al., 1995). The testing procedure employed two observers blinded to the treatment watching unrestricted animal ambulation in a wide testing arena for 4 minutes. The animal's functional performance was graded on a 21 point scale and was based on the extent of hind limb movement, weight-support, stepping and coordination, toe clearance and tail position. A score of 0 indicated complete paralysis, while a score of 21 indicated normal ambulation. BBB testing was performed on the 1st post-operative day and once weekly thereafter for 6 weeks with the last assessment performed on the day of the sacrifice. All testing was conducted in the morning, in the same room by the same two personnel. Function was evaluated in both hind limbs and then averaged for each animal.

Footprint analysis using the CatWalk system

The digital CatWalk System (Noldus Information Technology Inc, Leesburg, VA) was utilized to assess fine motor recovery and in-line ambulation. The system has been previously validated for evaluating motor recovery following dorsal hemisection SCI (Hamers et al., 2001). In short, animals were trained to cross an elevated glass plate with internal fluorescent illumination. The plate is designed to reflect all light internally,

however, once the animal touches the surface, light is reflected downward and recorded by a digital camera positioned below, attached to a computer. The reflection generated a distinct image of the foot-print and allowed for the whole run to be analyzed. Various ambulation data parameters were measured including the angle of paw rotation from the direction of walking, stride length, base of hind and fore limb support, paw swing duration, print area, duration of tail and abdominal dragging, etc.

Prior to surgery the animals were trained to cross the walkway, then the baseline data was obtained. The tests were repeated post-operatively on a weekly basis starting with 1 week after injury. All testing was performed in the morning, in the same room by the same personnel. Animals were allowed to rest for 1 hour between the Catwalk testing and open field locomotion assessment.

Tissue Collection and Preparation

At the respective endpoints (1 week or 6 weeks post lesion), all animals were deeply anesthetized with ketamine /xylazine and transcardially perfused with PBS followed by perfusion with ice cold 4% paraformaldehyde (PFA). The full-length spinal cord was then excised and post-fixed overnight at 4°C degrees. Tissue was cryoprotected in 30% sucrose in phosphate buffer (PBS) and maintained at 4°C for at least 72 hours or until sinking. Spinal cord sections extending ±5mm around the lesion, plus one rostral and one caudal section were collected, embedded in OCT compound, quick frozen on dry ice and stored at -80°C until needed. In animals injected with mini-ruby, spinal cord sections around the lesion were cut at 3mm rostral and 12mm distal to the site of the hemisection. Additional rostral and distal spinal cord samples (10mm long each) were also collected, embedded in OCT compound and stored at -80°C for later sectioning and

axonal counting. Serial 20μm sagittal sections of spinal cord were generated by cryostat, mounted on gelatin-coated glass slides and stored at -80^{o}C until use. Six sections spaced at 320μm intervals across the lesion were mounted on each slide to provide evenly distributed sections for each antiserum.

Immunohistochemistry:

Prior to staining, spinal cord sections were re-hydrated in PBS and blocked for one hour at room temperature in 5% goat serum / 0.03%Triton X-100 in PBS. Appropriate primary antibodies were then added and tissues incubated overnight at 4°C. The following primary antisera were used: reactive astrocytes – rabbit polyclonal anti-GFAP (1:500, Dako, Denmark), macrophages/microglia – mouse monoclonal anti-ED-1 (1:175, Millipore, Billerica, MA), fibroblasts – mouse monoclonal anti-vimentin (1:20, Sigma Aldrich, St.Louis, MO), chondroitin sulfate proteoglycans – mouse monoclonal anti-CS56 (1:200, AbD Serotec, Raleigh, NC), rabbit polyclonal anti-NG2 (1:500, Millipore, Billerica, MA), mouse monoclonal anti-neurocan (1:500, Millipore, Billerica, MA). After three washes in PBS, sections were incubated for 90 minutes with the appropriate secondary antibodies conjugated either to Alexa-488 or Alexa-568 (goat anti-rabbit and anti-mouse, respectively) (1:200, Molecular Probes, Eugene, OR). Slides were allowed to dry and were coverslipped with DAPI-containing mounting medium to label nuclei. To control for non-specific secondary antibody binding, a primary antibody omission control was included with each batch of slides stained. For each antibody, staining of all sections from all groups was performed on the same day using the same batch of diluted antibody.

Serial 20μm longitudinal sections along the coronal plane were obtained by cryostat from spinal cord samples of animals injected with mini-ruby and mounted on gelatin-coated glass slides for axonal counting. Every sixth section was collected starting from the dorsal surface of the spinal cord until the level of the gray commissure was reached. All sections were co-stained with GFAP and detected with Alexa-488 conjugated goat anti-rabbit antiserum as above to enable precise identification of the lesion center.

Quantitative Analysis:

Immunohistochemistry

Immunofluorescent analysis of tissue staining with each antibody was performed on the spinal cords of at least four animals randomly selected from each treatment group. Similar to the staining procedures, image acquisition for a specific antibody for the two groups at each survival time-point was performed on the same day. Six spinal cord sections per animal, separated by 320μm, were digitally photographed using an Olympus BX61 microscope with an attached CCD camera. Depending on signal intensity of a particular antibody, images were obtained at a magnification of either 2X (single image) or 4X (two images: left and right of the lesion). This allowed for the quantification of the relative immunofluorescence intensity of each antibody within a 2mm circumference surrounding the lesion center. For each antibody the image acquisition settings remained constant for all spinal cord sections analyzed. Following image acquisition the relative intensity of antibody labeling within the pre-defined area was quantitated using iVision-Mac™ Software (Bio-Vision Technologies, Exton, PA). Immunofluorescence intensity

was calculated using the threshold method. The threshold value for positive staining was set and pixel intensity exceeding that level was quantified. The threshold remained constant for spinal cord sections from all treatment groups stained with the respective antibody. For the 2X images pixel intensity values obtained for the six sections from the same animal were tabulated and averaged. For images obtained at 4X, pixel values from the right and left side of the lesion were first added for each section and then averaged for the six sections from each animal. Spinal cord sections that were folded or damaged while transferring from the cryostat onto the slide were excluded from the computational analysis. The data are presented as means ± standard error of the mean (SEM).

Anterograde Axonal Labeling Assessment

Mini-ruby labeled axons were counted in two sections per animal. Axonal counting was performed at 0.25mm intervals (magnification 40X) that extended 5mm rostral from the lesion center and 10mm in the caudal direction. The lesion center was identified as an area devoid of GFAP immunoreactivity but filled with DAPI-positive cell nuclei. All tissue analysis was performed using the Olympus BX61 microscope. Total axon numbers rostral to the lesion and caudal to it as well as average axon lengths were calculated as previously described (Byrnes et al., 2005; Wu et al., 2009). All data are reported as means ± SEM.

Micro Computed Tomography (MicroCT)

In the 6 week follow-up group, all spinal columns were radiographically evaluated using a microCT system (SkyScan 1172, SkyScan, Inc., Belgium). Following tissue perfusion with 4% PFA each spinal column was excised *en bloc* and transferred to the CT scanner. Scans were performed at an 11μm resolution and images reconstructed in

the sagittal, coronal and axial planes for analysis. The extent of bone formation into the spinal canal and neural foramina were qualitatively evaluated.

Data and Statistical Analysis

Numerical data are presented as mean ± SEM. Intergroup differences between control and rhBMP-2 treated animals were compared using a Student's t-test at each follow-up point. At 1 week post lesion (wpl), intergroup comparison of the inflammatory response between the three groups were evaluated using an one-way analysis of variance (one-way ANOVA) followed by the Tukey's Honestly Significant Difference (HSD) test as post hoc comparison. All statistical computations were performed using the SPSS 16.0 software (SPSS, Inc., Chicago, IL) and a difference at $p<0.05$ was considered significant.

RESULTS

Model of spinal cord injury and rhBMP-2 application

We have previously shown that rhBMP-2 applied posterolaterally on a collagen carrier in the vicinity of a dorsal hemisection SCI activates a functional signaling cascade in all cell types of the damaged spinal cord 24 hours after application. SCI was induced via a dorsal hemisection at T9 to generate the worst-case scenario for exposing the spinal cord parenchyma to the exogenous protein. We therefore continued with this model of SCI. As surgical decompression is advocated within the first 8 to 24 hours after SCI clinically, we modeled clinical practice by implanting the collagen sponges with or without rhBMP-2 30 minutes after dorsal hemisection, (deemed appropriate due to the differential metabolic rates between the animal model and humans) (Fehlings and Perrin, 2006).

Morphologic characterization of the lesion

Effects of rhBMP-2 on the composition of the lesion scar at 1wpl

One week following dorsal hemisection and ACS implantation to the spine significant changes in the morphology of the lesioned spinal cord were observed between the control animals and those receiving rhBMP-2. rhBMP-2 triggered an increased intrathecal inflammatory response. ED-1 staining of the parasagittal sections surrounding the lesion indicated significantly greater levels of the infiltrating macrophages and endogenous activated microglia near the injury site (Figure 1A). rhBMP-2 treatment resulted in an increase in the intensity and area of ED-1 staining around the lesion compared to either animals implanted with vehicle controls (84% increase) or with

recombinant human albumin (81% increase) (ANOVA; F=5.56, p=0.027). In contrast, ED-1 immunolabeling was similar between the vehicle and albumin controls, indicating that implantation of a non-specific human protein did not produce a significant change in the post-injury inflammatory response.

As astrocytes and fibroblasts express BMP receptors and respond to BMP, we examined the lesioned spinal cords for markers of reactive astrogliosis (GFAP) and intermediate filament of the fibroblast cytoskeleton (vimentin) (Gomes et al., 2003; Hampton et al., 2007; Huang et al., 2007). Reactive astrocytes upregulate expression of both GFAP and vimentin, whereas fibroblasts express only vimentin. *T*hus, we identified fibroblasts as cells that were vimentin positive, but GFAP negative providing a mechanism of quantifying the fibroblast infiltration in the lesion (Conrad et al., 2005). Immunofluorescent analysis of the stained sections around the lesion revealed significantly greater labeling with both GFAP and vimentin in the rhBMP-2 group compared to the control group (Figure 2). The extent of reactive astrogliosis in the vicinity of the lesion was 181% higher in the rhBMP-2 group relative to that of the vehicle control (t-test; p=0.002). rhBMP-2 also stimulated intraparenchymal fibroblast invasion and ectopic fibrous scar formation. In the spinal cords of rhBMP-2 treated animals GFAP negative, vimentin positive labeling was 157% greater than in the vehicle control animals (t-test; p=0.021).

Following spinal cord injury, the cells within the fibroglial scar are known to produce a number of extracellular matrix molecules including various chondroitin sulfate proteoglycans (CSPGs), which are highly inhibitory to axonal regeneration (Miller and Silver, 2006). Therefore, we probed the lesioned tissues with a CSPG antibody (CS56)

that recognizes several different epitopes on the intact glycosaminoglycan side-chains of the proteoglycan molecule (Avnur and Geiger, 1984; Ito et al., 2005). CSPG immunoreactivity was doubled in the area surrounding the lesion in rhBMP-2 treated animals in comparison to control animals (t-test; p=0.04) (Figure 1B). To further characterize the molecular composition of the glial scar, we examined the injured spinal cords for two specific CSPG core proteins NG2 and neurocan. Both fibroblasts and macrophages/microglia express NG2, whereas neurocan is expressed by astrocytes (Fawcett, 2006). We observed a strong trend for the increased NG2 immunoreactivity within the glial scar in animals receiving rhBMP-2 but this did not reach statistical significance (80% labeling increase; t-test; p=0.17) (Figure 1C). In contrast, neurocan staining was similar for both groups with only a marginal 12% difference between the rhBMP-2 and the control groups (t-test, p=0.699).

Chronic effects of rhBMP-2 on the composition of the lesion scar at 6wpl

Following the 6 week survival period, all spinal columns from the animals in the rhBMP-2 group were radiographically imaged using the microCT to evaluate the status of the posterior fusion mass prior to spinal cord collection. Manual palpation during the gross dissection and subsequent radiographic evaluation indicated that spinal fusion was achieved in 100% of cases. Further microCT reconstructions in the axial, sagittal and coronal planes revealed no cases of bone encroachment into the spinal canal with all fusion masses contained dorsal to its circumference (Figure 3). Therefore, at 6wpl no morphologic and or behavioral differences observed between the rhBMP-2 treated and

control animals were attributed to the mechanical compression of the spinal cord secondary to ectopic bone formation.

Immunohistochemical analysis of the longitudinal spinal cord sections surrounding the lesion epicenter showed that most of the changes caused by rhBMP-2 treatment at 1wpl were still apparent at 6 weeks post lesion, although often with a reduction in the magnitude of differences between the two treatment groups (Figures 4-5). We observed a strong trend for increased intrathecal macrophage/microglia immunoreactivity (167% of control) in the vicinity of the lesion in rhBMP-2 treated animals at 6 weeks post lesion. However, these differences were not statistically significant (t-test; $p=0.157$) (Figure 4A). Similarly, GFAP immunolabeling was 51% greater in rhBMP-2 treated animals compared to control animals. Due to low within group variability these differences were statistically significant (Student t-test $p=0.021$) (Figure 5). The extent of fibroblast invasion in the scar in the rhBMP-2 group was also lower at 6wpl than at 1wpl. At 6 wpl there were no significant differences in anti-vimentin immunoreactivity in GFAP negative cells in tissues exposed to rhBMP-2 compared to the spinal cords from the vehicle control group (t-test; $p=0.297$).

Despite the reduction in fibroblasts in the scar at 6 wpl, a strong trend for increased immunolabeling with the anti-CSPG CS-56 antibody persisted in the rhBMP-2 treatment group (t-test; $p=0.111$) (Figure 4B). Furthermore, NG2 immunoreactivity was significantly greater at the lesion epicenter and the adjacent spinal cord tissues in the rhBMP-2 group, highlighting chronic effects of rhBMP-2 on CSPG deposition (t-test; $p=0.031$) (Figure 4C). However, as observed at the 1wpl time-point, there were no

intergroup differences in neurocan immunofluorescence at the long-term follow-up (t-test; p=0.759).

Anterograde Axonal Tracing

To identify re-growing axons of the corticospinal tract (CST) in rats after SCI and implantation of either control or rhBMP-2 soaked sponges we injected an anterograde tracer (mini-ruby) into the primary motor cortex 10 days before sacrifice. Following spinal cord sectioning in the coronal plane, the injected fluorescent dye was clearly visible in areas rostral to the lesion. It was localized within the descending axons of the dorsal column white matter, corresponding to the CST tract in the rat (Hodgetts et al., 2009) (Figure 6A). Immediately proximal to the lesion, dense areas of axonal sprouting were observed within the dorsal column (Figure 6B). Interestingly, throughout this zone we identified marginally greater numbers of axonal sprouts in the rhBMP-2 treated animals compared to the controls; however, high within group variation precluded statistical significance (1740±724 vs. 909±198, respectively; p=0.31) (Figure 7A). In contrast, despite the low overall counts for both groups, distal to the injury epicenter we observed a strong trend for spontaneous regeneration in the control group (Total axon counts: Control= 270±45; rhBMP-2=156±25; p=0.07) (Figure 7B). With respect to the average length of axonal growth, there were no appreciable differences between the two groups.

Behavioral Testing of Locomotor Function

Open Field Locomotion

Open field ambulation and return of locomotor function was monitored using the BBB locomotor rating scale. All animals were tested pre-operatively, on the 1st post-

operative day and once weekly thereafter. On the 1st post-operative day rats in all three groups (rhBMP-2, rhAlbumin and vehicle control) received identical scores indicating consistency of the SCI induction among the treatments (BBB scores: 10.8 ± 0.75; 10.4 ±0.63; 10.8 ±0.98, respectively) (Figure 8). In contrast, BBB testing at 1wpl indicated that rats implanted with rhBMP-2 had significantly worse motor function than rats implanted with control sponges (t-test; $p<0.05$). Rats implanted with rhAlbumin on the ACS demonstrated identical recovery to the vehicle control group (BBB scores: 13.6 ± 1.02 and 13.5 ± 0.48, respectively). However, at later time-points motor function, as assessed by the BBB rating, was similar in the control and the rhBMP-2 treated groups with only appreciable differences recorded at the 3wpl and 4wpl time points ($p>0.05$).

Footprint Gait Analysis using the CatWalk System

To gain a more complete and detailed analysis of motor function, in-line ambulation and fine motor deficits were assessed using the digitized CatWalk system. All animals were trained pre-operatively to traverse a horizontal glass plate followed by baseline pre-injury recording. All post-injury data was then normalized to the baseline values for each animal and presented as percent change. Footprint analysis was not performed on the 1st post-operative day as the rats were unable to even partially step, preventing analysis. Day 7 testing however, revealed significant differences in the change of the hindlimb angle of paw rotation from the midline, with rats in the rhBMP-2 group demonstrating a 318% increase in paw exorotation, an indication of a loss in fine motor control, compared to a 47% increase in the control group ($p=0.019$) (Figure 9A). Interestingly, at day 14, fine motor control in the rhBMP-2 treated animals improved compared to the earlier time-point and approximated that of the control group

(188%±39% vs. 154±29%, respectively). This improvement in the angle of paw rotation was not permanent and additional changes in the parameter were recorded at the later time-points, whereas in the control group the initial post-injury change was maintained throughout the 6 week survival period. At the time of the latest assessment (6wpl) the differences between the two groups were significant: we recorded a 473% change in the paw rotation angle in the animals treated with rhBMP-2 versus 66% change in the control group (t-test; $p=0.048$).

Additional differences in animal ambulation were observed while comparing the percent increase in the base of support of the hind limbs (Figure 9B). In concurrence with other findings, there was a significantly greater increase in this parameter in the rhBMP-2 group relative to the controls at the 1wpl assessment (t-test; $p=0.003$). Similar trends persisted at 2wpl, however, were diminished at the later time-points ($p>0.05$).

No other comparisons between the two groups including stride length, swing duration, contact area or duration of tail and abdominal drags were statistically different at any follow-up time-points ($p>0.05$).

DISCUSSION

The FDA approval of rhBMP-2 for the treatment of discogenic pathology in the lumbar spine provided a new direction for the science of autogenous bone graft substitutes. Considerable clinical experience with the protein has expanded the spinal pathology managed through arthrodesis with rhBMP-2. Additional clinical indications, however, have uncovered complications associated with the biologic substitute that were not seen in the original clinical study. More recently, anecdotal reports of managing segmental instability with or without neurologic compromise through rhBMP-2 induced fusion have emerged. Based on the paucity of basic science data on the direct effects of the protein on the spinal cord, clinical application of rhBMP-2 for this indication may be premature. We, therefore, undertook a systematic evaluation of the acute and long term morphologic and functional changes triggered by a posterolateral arthrodesis with rhBMP-2 implanted in the immediate vicinity of a spinal cord lesion.

At 7dpl we observed a pronounced increase in the macrophage/microglial staining in animals receiving rhBMP-2 compared to those treated with vehicle control (Figure 1A). Invading macrophages and activated resident microglia are known mediators of post-SCI inflammation (Batchelor et al., 2008). They contribute to secondary cell death through release of free radicals, inflammatory cytokines such as tumor necrosis factor α (TNF-α) and nitric oxide (NO) (Hausmann, 2003). Increased inflammation may be detrimental to recovery from SCI. Indeed, there was a significant worsening of ambulatory performance by animals in the rhBMP-2 group compared to the controls one week post lesion, despite comparable BBB scores observed on the 1st post-operative day between the groups. Thus, treatment with rhBMP-2 led to a transient

worsening of motor function that correlated with increased infiltration and activation of inflammatory cells around the lesion.

Concurrent with macrophage upregulation, spinal cords of animals treated with rhBMP-2 revealed increased labeling with GFAP, a marker of reactive astrocytes. After SCI reactive astrocytes play a critical role in the formation of a glial scar and production of inhibitory CSPGs that impede axonal regeneration through the lesion (Fitch and Silver, 2007). rhBMP-2 directly alters astrocyte hypertrophy and GFAP expression (D'Alessandro et al., 1994) and has also been shown to upregulate specific CSPG core proteins in astrocyte culture (Fuller et al., 2007). We observed a twofold increase of immunoreactivity for CSPGs around the lesion using the CS56 antiserum in the spinal cords of animals treated with rhBMP-2. This antiserum recognizes several different epitopes on the sulfated GAG chains of different core CSPGs (Avnur and Geiger, 1984), so it indicates an increase in the amount of GAG chain without specifying which core proteins may be involved in this deposition. BMP-4 was shown to increase the RNA for aggrecan, neurocan and brevican in astrocyte culture (Fuller et al., 2007). However, we found no overall difference in neurocan staining of the spinal cord between the two different experimental groups despite the increased GFAP expression in astrocytes in response to rhBMP-2. We did observe a strong trend for increased NG2 immunoreactivity surrounding the lesion in animals treated with rhBMP-2. This CSPG is often used as a marker for oligodendrocyte progenitor cells, suggesting that there may be an increase in the number of OPCs surrounding the lesion.

Meningeal fibroblasts invading the lesion form the core of the fibroglial scar, which constitutes a major physical barrier to axonal growth (Fawcett, 2006). In turn, they

produce several extracellular matrix molecules including semaphorin 3 and NG2 that further contribute to the non-permissive qualities of the lesion core (Shearer et al., 2003). Our data showed that rhBMP-2 contributed to a marked increase in tissue fibrosis and ectopic scar formation.

Similar trends in the composition of the fibroglial scar were seen at 6 weeks as at 7 days post lesion. Increased inflammation, GFAP immunoreactivity and NG2 deposition were evident in spinal cords from the rhBMP-2 treated animals as compared to the controls. These findings are important because initial studies with the ACS carrier sponges indicated that only 50% of the rhBMP-2 remains on the sponge at 10 days following implantation and all protein is metabolized/excreted by 4 weeks after implantation (FDA, 2002). Therefore, morphologic changes within the spinal cord observed at 6 weeks post-SCI, persist after all protein has been cleared from the surgical site. Our data suggest that long term changes in spinal cord morphology result from the transient use of rhBMP-2 around the injury site.

Detailed microCT analysis of bone indicated solid fusion masses in all rats treated with rhBMP-2. Our primary concern was to remove the possibility that rhBMP-2 induced bone would encroach into the spinal canal and cause mechanical compression of the spinal cord. By modifying the surgical technique we were able to prevent this encroachment. Instead of creating a complete laminectomy of either T9 or T10 as described in a more classic approach, we performed the dorsal hemisection through the interlaminar space of T9-T10. Using this technique, the interlaminar space was distracted to access the spinal cord and then re-approximated following clamp release from the adjacent spinal processes. MicroCT analysis showed no evidence of bone formation in

the spinal canal in any animals and allowed us to conclude that all morphologic and functional changes observed at 6 weeks post lesion resulted from the direct effect of rhBMP-2 on spinal cord tissue rather than chronic compression of the spinal cord.

Anterograde labeling of the descending fibers comprising the CST tract was performed to analyze whether rhBMP-2 had an effect on axonal regeneration. Dorsal hemisection SCI results in the complete transection of the descending fibers of the CST tract; therefore, axons labeled with the fluorescent dye distal to the lesion result from spontaneous regeneration. The direct effects of rhBMP-2 on neuronal growth are not fully understood. Inhibition of endogenous BMP signaling within the spinal cord by intrathecal infusion of the BMP-specific antagonist, noggin, resulted in improved functional recovery and CST axon growth following an SCI (Matsuura et al., 2008). In contrast, Zou et al (Zou et al., 2009) found that axotomy induced Smad-1 upregulation increased axonal growth in adult sensory neurons.

In the current project, CST axonal sprouting in the BMP group was moderately increased rostral to the lesion; however, axonal numbers distal to the injury were markedly reduced relative to the control SCI group. This observation requires further study as uncontrolled axonal sprouting could result in improper synapse formation in the dorsal horn and post-injury onset of neuropathic pain. In the rat, afferent sensory axons synapse on spinal interneurons in laminae 1, 2, whereas descending CST fibers terminate on the respective lower motor neurons, which are found in laminae 3-6 (Hodgetts et al., 2009). Improper signaling could facilitate progression of the chronic pain syndrome, a frequent post-SCI complication reported clinically and in animal models (Sjolund, 2002; Hulsebosch, 2005; Nesic et al., 2005). A profound manifestation of neuropathic pain in

the spinal cord injured rats is the development of autophagia. Interestingly, in our study this phenomenon was noted in 56% of rhBMP-2 treated rats versus only 35% of the control animals. Whether rhBMP-2 elicited this response through increased sprouting or other mechanisms remains to be determined; however, macrophages and reactive glia are known modulators of mechanical allodynia (Stuesse et al., 2001; Ji and Suter, 2007). Thus rhBMP-2 could have an indirect effect on post-injury allodynia through upregulation of macrophage infiltration and astrocyte reactivity. However, rhBMP-2 could also have a direct effect on the expression of various neuropeptides regulating pain in the dorsal horn and dorsal root ganglia. Clinically, post-operative radiculitis is one of the recently reported post-operative complications in patients undergoing transforaminal lumbar interbody fusion (TLIF) with rhBMP-2 (Rihn et al., 2009). Therefore, additional studies are necessary to fully elucidate the exact role and mechanism of rhBMP-2 on the different sensory and motor neurons of the spinal cord, as well as its modulation of pain.

Functionally, we observed significant differences in the degree of hind paw exorotation between the control and rhBMP-2 treated animals at 6 weeks post lesion. Unlike in humans, the corticospinal tract (CST) lesion in a rat does not preclude animals from spontaneously regaining function of the hind limbs, including locomotion (Hodgetts et al., 2009). Instead, CST lesions are associated with the loss of fine motor skills, including paw position control (Muir and Whishaw, 1999). Therefore, rhBMP-2 had a significant detrimental effect on the functional motor skills assessed by measuring changes in the hind paw angle of rotation. BBB testing, although repeatedly validated over the years for assessing functional recovery following a contusion SCI, was not sensitive enough to delineate functional changes in our study. Nevertheless, we did

observe significant worsening of functional performance on the BBB rating at one week after injury that corresponded to the peak inflammatory response elicited by rhBMP-2.

In conclusion, these results demonstrate that spinal arthrodesis with rhBMP-2 performed in the vicinity of a SCI has extensive negative effects on the course of spinal cord pathology. Treatment with rhBMP-2 resulted in increased intrathecal inflammation, gliosis, fibrous scarring and deposition of inhibitory CSPGs, which may limit axonal regeneration. Ultimately, this response contributed to decreased functional recovery at 6 weeks post lesion. Although performed in a rat model of dorsal hemisection SCI, our findings suggest that the use of rhBMP-2 may not be the optimal choice in patients with spinal cord pathology or significant dural deficiencies.

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FIGURES

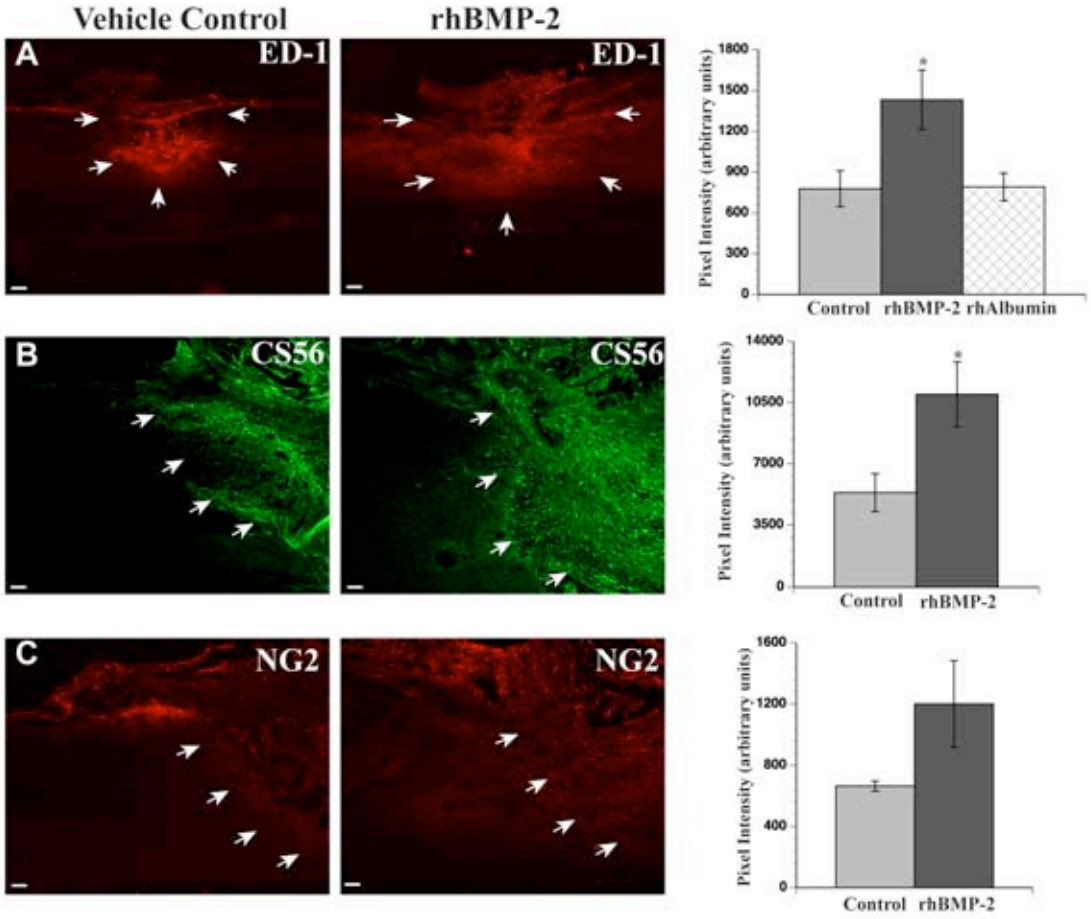

Figure 1: rhBMP-2 implantation to the spine triggered significant morphologic changes within the spinal cord after 1 week. (A-C) Representative sections from control and rhBMP-2 treated animals showed that spinal cords from rats treated with rhBMP-2 had elevated levels of ED-1 (A), CS56 (B) and NG2 (C). Arrows indicate (A) the outline of the lesion or (B and C) the rostral border of the lesion. Quantification of these changes indicated that (A) there was a significantly greater ED-1 staining in the rhBMP-2 group compared to the carrier alone and rhAlbumin control groups (bar graph; $p<0.05$). (B) Similarly, rhBMP-2 implantation contributed to a significant increase in the total CSPG production in the vicinity of the lesion ($p<0.05$). (C) NG2 immunoreactivity was also increased; however, these differences were not statistically significant ($p=0.17$). All data represent mean ± SEM (n= 4). (A) Scale bar 200μm, (B-C) scale bar 100μm.

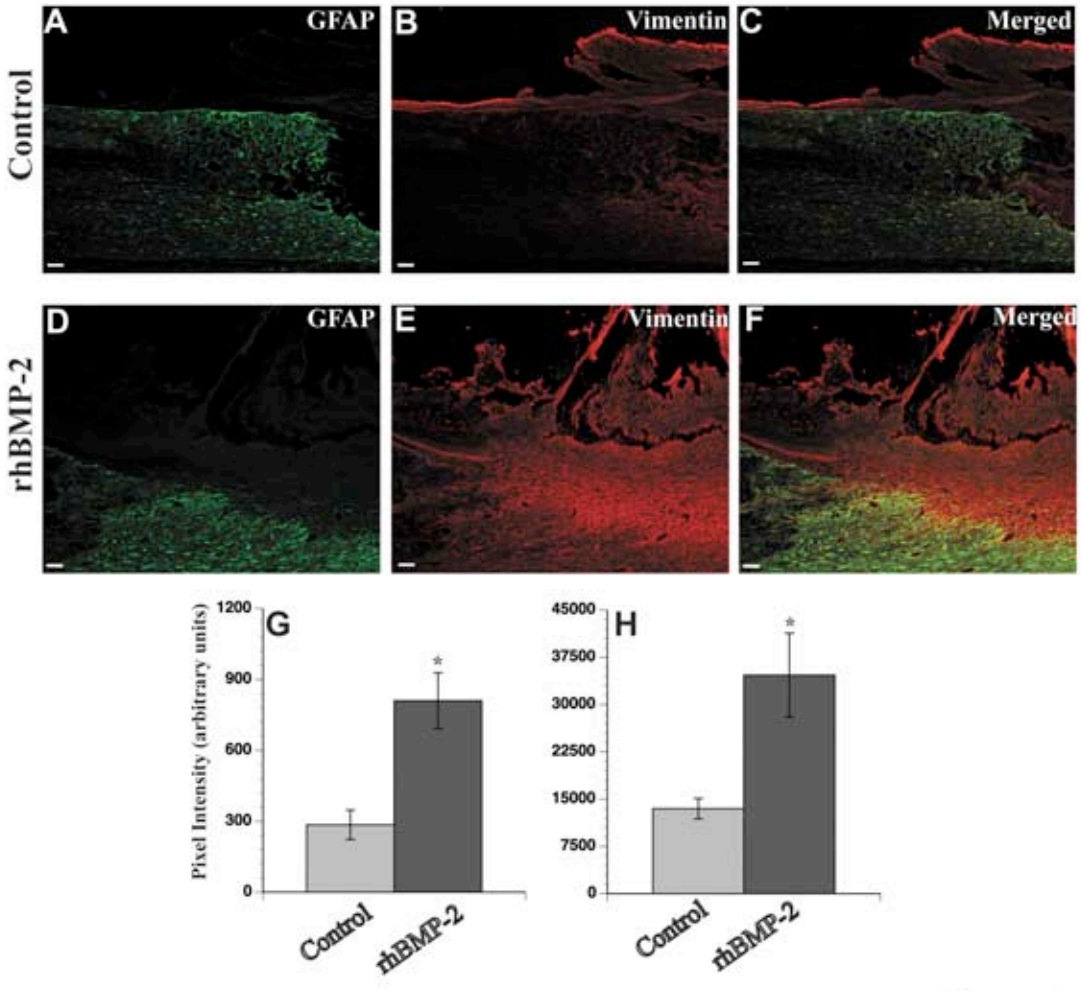

Figure 2: GFAP and vimentin immunolabeling are increased in the spinal cord of rhBMP-2 treated animals at 1 week post lesion. Representative longitudinal spinal cord sections from the 1wpl control (A-C) and rhBMP-2 (D-F) groups showing GFAP and vimentin distribution near the lesion. (H) GFAP immunolabeling was significantly increased in the rhBMP-2 treatment compared to the control group ($p<0.05$). (I) rhBMP-2 application also contributed to the increased fibrotic scarring as indicated by the area of GFAP negative vimentin positive immunostaining ($p<0.05$). All data represent mean ± SEM (n= 4). Scale bar 100μm.

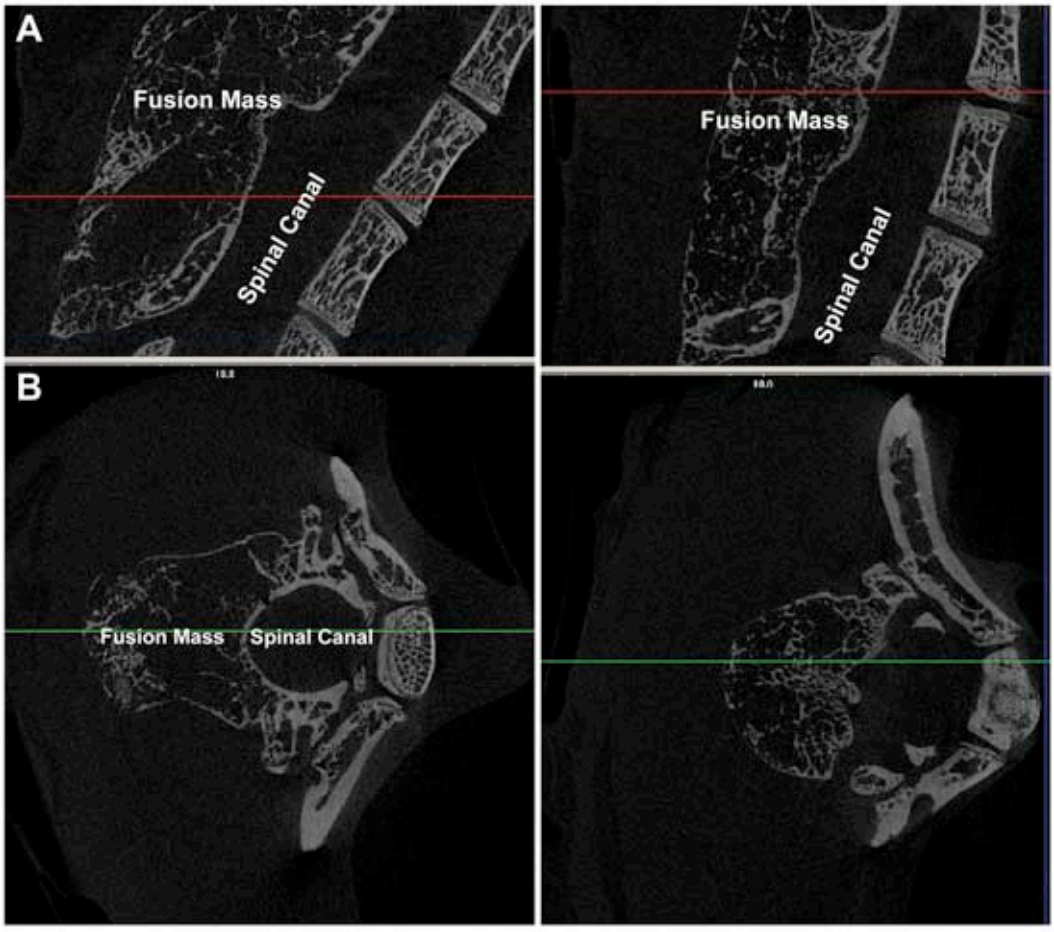

Figure 3: MicroCT scans of the rhBMP-2 treated spinal columns at 6wpl show no bone formation within the spinal canal. Radiographic fusion was achieved in all animals treated with rhBMP-2 with all fusion masses contained dorsal to the spinal canal. Sagittal (A) and axial (B) scans from two separate rhBMP-2 treated rats are shown. Resolution 10μm.

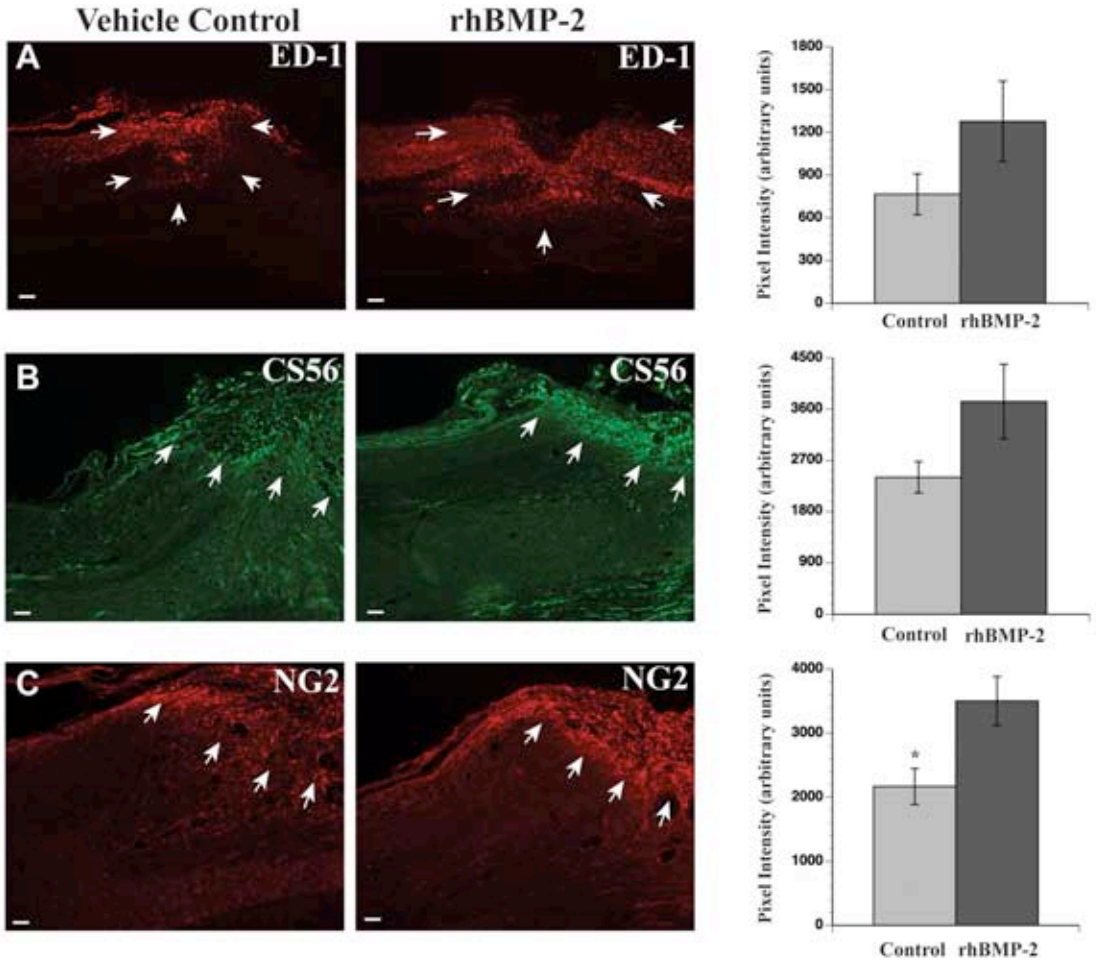

Figure 4: Distribution of ED-1, CS56 and NG2 immunolabeling around the lesion core at 6 weeks after injury. Sagittal spinal cord sections from rats treated with rhBMP-2 show strong trends for increased ED-1 (A) and CS56 (B) immunoreactivity ($p=0.157$ and $p=0.111$, respectively). (C) NG2 immunoreactivity was significantly stronger in the rhBMP-2 group compared to the control samples ($p=0.031$). All data represent mean ± SEM ($n=4$). (A) Scale bar 200μm, (B-C) scale bar 100μm.

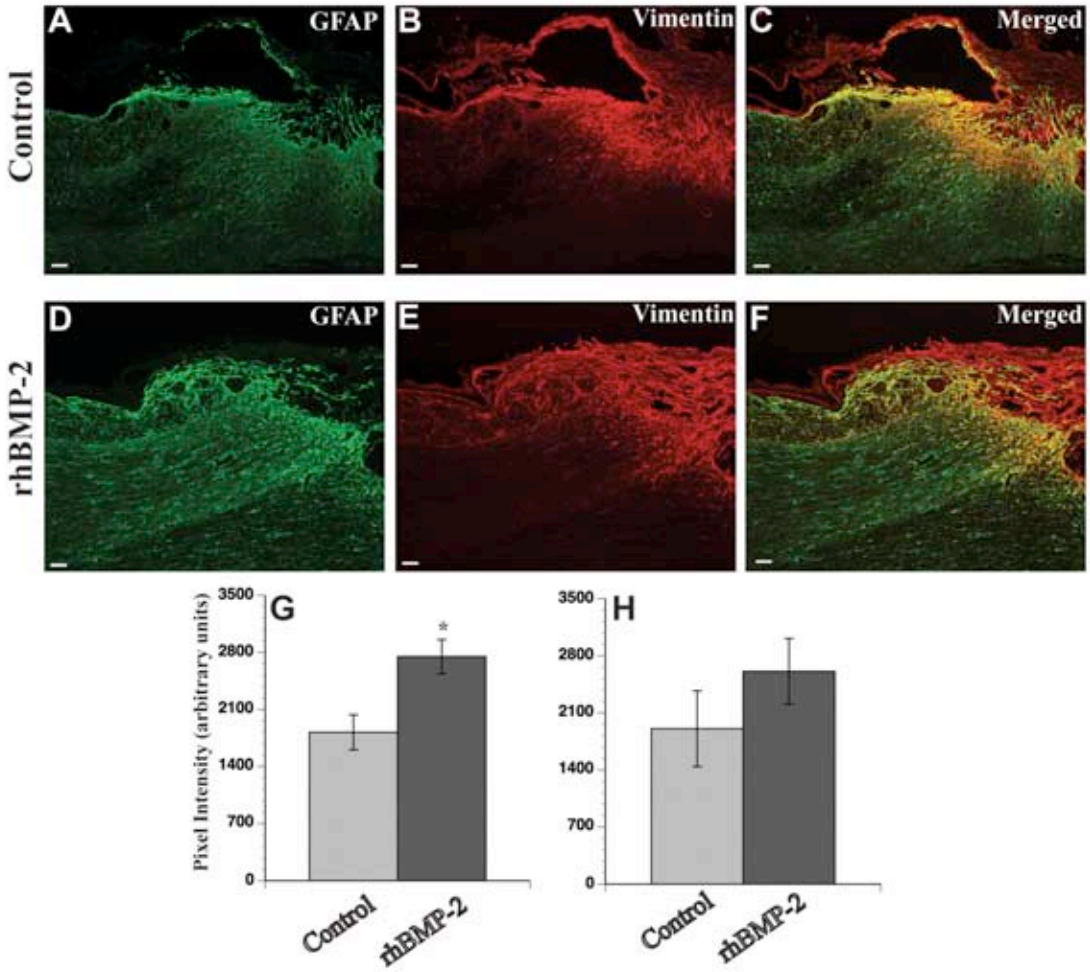

Figure 5: GFAP immunolabeling is increased in the spinal cord of rhBMP-2 treated animals 6 weeks post lesion. Representative longitudinal spinal cord sections from the 6 wpl control (A-C) and rhBMP-2 (D-F) groups showing GFAP and vimentin immunostaining around the lesion. (H) GFAP immunolabeling was significantly increased in the rhBMP-2 treatment compared to the control group ($p<0.05$). (I) Intergroup differences in the GFAP negative vimentin labeling were reduced at 6wpl compared to 1wpl; however, a trend for increased fibrotic scarring in the rhBMP-2 group was maintained ($p>0.05$). All data represent mean ± SEM (n=4). Scale bar 100μm.

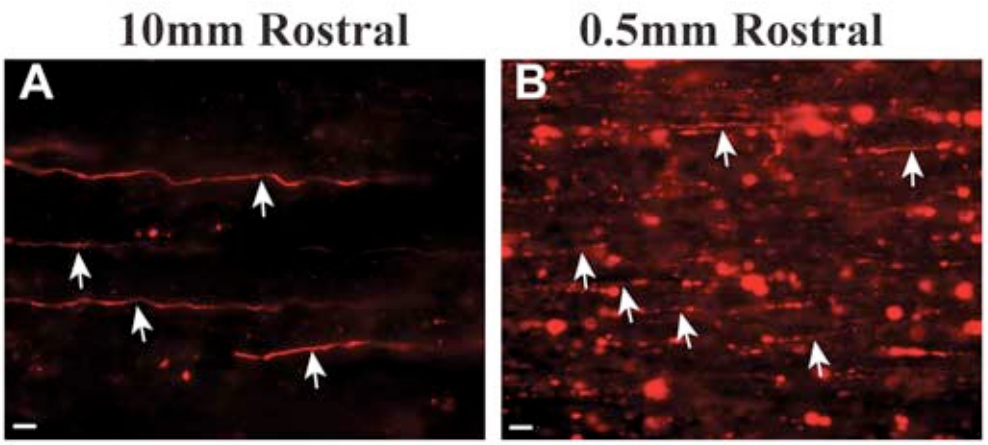

Figure 6: Axonal tracing of corticospinal neurons from the motor cortex. Longitudinal coronal section through the white matter 10mm rostral (A) and immediately adjacent (B) to the lesion shows many more sprouting neurons in the vicinity of the lesion. (A) Note continuous axons (red, arrows) labeled with mini-ruby. (B) Axonal sprouts (arrows) observed within the white matter adjacent to the lesion core, with irregular staining and multiple short sprouts terminating at the rostral end of the lesion. Scale bar 10μm.

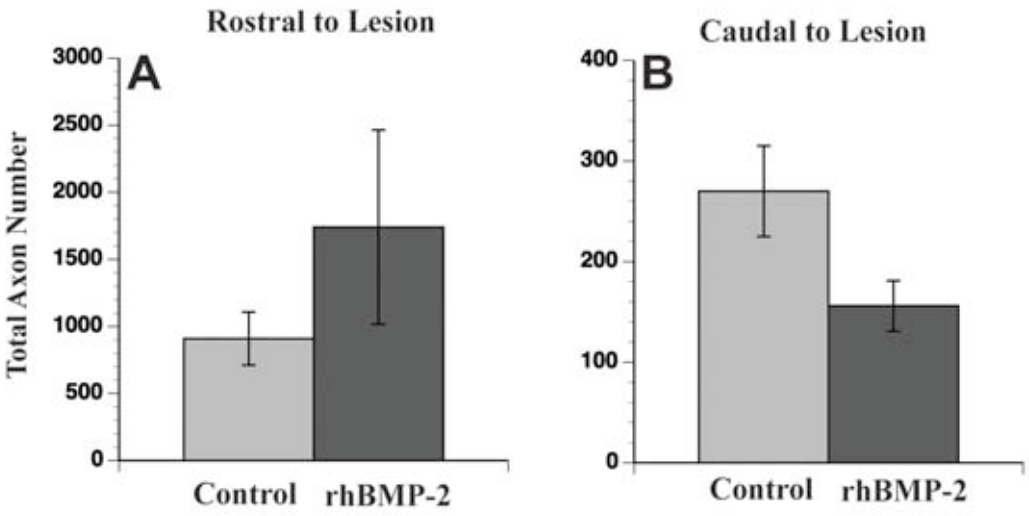

Figure 7: Quantification of the mini-ruby positive corticospinal axons within the white matter columns. (A) Total number of axonal sprouts counted within 1mm immediately rostral to the lesion border. We observed a trend for increased axonal sprouting rostral to the lesion in spinal cords from the rhBMP-2 treated animals relative to animals treated with vehicle control ($p=0.31$). (B) Total number of axons observed within 10mm distal to the lesion. Despite the low overall counts for both groups, there was a strong trend towards less spontaneous regeneration in those animals treated with rhBMP-2 than in control animals ($p=0.07$). All data represent mean ± SEM ($n=8$).

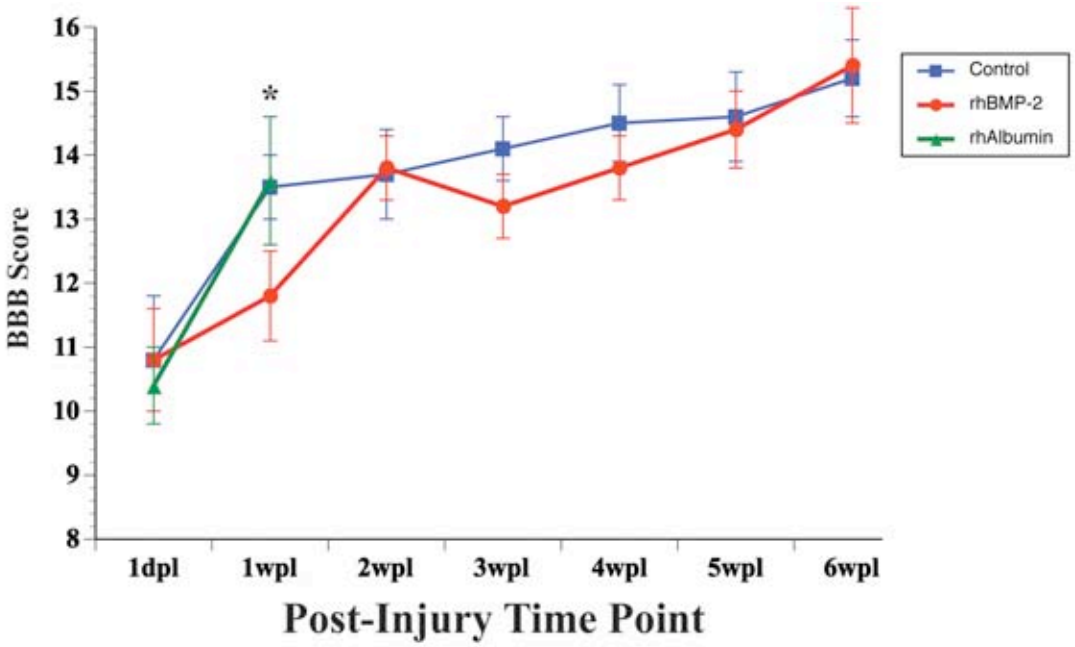

Figure 8: Functional recovery of locomotor function determined by BBB scoring. Animals in all three groups demonstrated similar functional deficits at 1dpl. By 1wpl, however, rhBMP-2 treated animals were functionally impaired relative to the two control groups of rats: those treated with vehicle control or with rhAlbumin ($p<0.05$). BBB scores in rhBMP-2 treated animals improved by 2wpl, but were marginally lower than the control group averages at 3- and 4-weeks post-injury. At 5 and 6 wpl locomotor function was similar between rhBMP-2 and vehicle control treated animals, All data represent mean ± SEM (n=16).

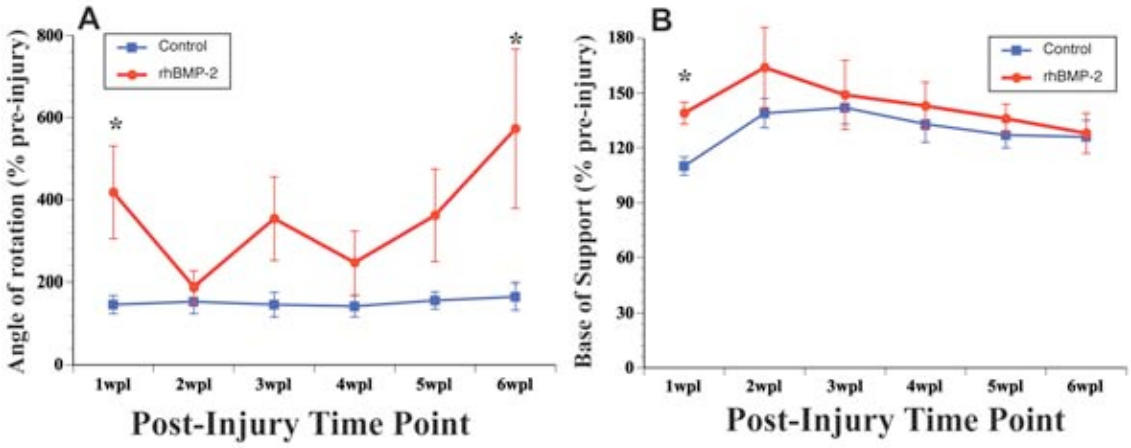

Figure 9: Functional recovery of ambulatory function according to analysis of footprint data with the Catwalk system. (A) Animals treated with rhBMP-2 showed significantly greater changes in the angle of hind paw exorotation relative to their pre-operative values than control group rats at 1wpl and at 6wpl ($p<0.05$). These data indicate that implantation of rhBMP-2 exerted long term detrimental effects on the spontaneous return of fine motor control of the hind limbs. (B) Rats treated with rhBMP-2 showed a greater increase in the base of hind limb support than the control animals at 1 wpl relative to their pre-operative values ($p<0.05$); however, this parameter did not show significant differences at later time points. All data represent mean ± SEM (n=8).

CHAPTER 4: DISCUSSION

Clinical use of rhBMP-2 in spinal surgery

The advent and commercialization of rhBMP-2 for clinical orthopaedic applications has opened a new chapter in our understanding of bone formation and repair. Numerous basic science and clinical studies have been conducted while establishing the mechanism of action, pharmacokinetics, safety and efficacy profiles of rhBMP-2 (Ebara and Nakayama, 2002; Valentin-Opran et al., 2002). Much of this work, however, has focused on direct bone induction and the ability of the exogenous rhBMP-2 to trigger mesenchymal cell differentiation along the osteoblastic lineage (Ryoo et al., 2006). Clinical studies have further confirmed the efficacy of rhBMP-2 in stimulating *de novo* bone formation in interbody fusion procedures and fracture repair of the appendicular skeleton (Boden et al., 2000; Govender et al., 2002). Improved rates of healing and limited complications have prompted the FDA in 2002 to approve the use of rhBMP-2, packed inside an interbody fusion device, for treatment of discogenic disorders in the lumbar spine (FDA, 2002). Since then, the use of rhBMP-2 as a substitute for autogenous bone in spinal procedures continues to gain in popularity among orthopaedic and neurological surgeons. Increased clinical experience has led to the "off-label" extension of original indications to include pathology of the thoracic and cervical spine. More recently, rhBMP-2 traumatic segmental instability with or without concomitant spinal cord pathology has been addressed through rhBMP-2 induced posterolateral arthrodesis.

Despite the overwhelming success in achieving spinal fusion reported in earlier studies, "off-label" indications revealed a number of significant post-operative

complications attributed to the exogenous protein. Major attention has been given to the soft-tissue swelling in the anterior cervical spine, as well as heterotopic bone formation into the spinal canal following a transforaminal interbody fusion in the thoracic region (Shields et al., 2006; Joseph and Rampersaud, 2007). Soft tissue inflammation and uncontrolled swelling in the neck can lead to grave consequences as a result of airway occlusion and requires immediate patient intubation. The risk of associated mortality prompted the FDA to issue a warning in July 2008 with regards to the use of rhBMP-2 in the cervical spine. With respect to the spinal cord, the only reports suggesting post-operative neurologic deterioration have been attributed to the heterotopic bone formed in the spinal canal that caused mechanical compression of neural elements (Wong et al., 2007). More recently, however, Rihn and colleagues described increased rates of post-operative radiculitis in patients undergoing transforaminal lumbar fusion with rhBMP-2, suggesting direct effects of the exogenous protein on the pathways of the spinal cord (Rihn et al., 2009). These complications and reports of using rhBMP-2 for spinal arthrodesis in patients with spinal cord trauma have prompted our studies, as there is a surprising paucity of basic science data on the direct effects of the exogenous rhBMP-2 on the cells comprising the spinal cord.

The goals of current research were to twofold: First, we examined whether exogenous rhBMP-2 applied over the spinal column can trigger a functional intrathecal signaling response in the intact and injured spinal cord. In this part of the project we also characterized the extent of rhBMP-2 induced intrathecal signaling as a function of post-SCI BSCB and meningeal barrier permeability. Once we established that rhBMP-2 does indeed elicit a downstream signaling cascade within the spinal cord, we carried out a

comprehensive evaluation of the acute and long-term effects of the protein on the composition of the glial scar, axonal regeneration and, ultimately, functional recovery.

Intraparenchymal penetration of exogenous rhBMP-2 and direct signaling activation

Clinical cases of high-energy spinal trauma frequently involve dural and spinal cord lacerations (Bellabarba et al., 2006). Therefore, we chose this model of SCI as it simulates the worst-case scenario of exposing the spinal cord parenchyma to the exogenous protein. Early implantation of rhBMP-2 (up to 7dpl) resulted in a significant increase in the number of pSmad positive cells in comparison to implantation of the water-containing ACS, showing that rhBMP-2 was indeed able to penetrate the spinal cord parenchyma and elicit a biological response. To our knowledge this was a first study documenting a profound intrathecal signaling response secondary to rhBMP-2 use in posterolateral arthrodesis in the presence of an SCI.

Our results indicated that all major CNS-resident cell types are responsive to BMP signaling. Nuclear pSmad staining was observed in cells co-labeled with specific markers for neurons, astrocytes and oligodendrocytes. Other cell types that were positive for pSmad include macrophages and activated microglia, as well as invading fibroblasts. With respect to the number of cells positive for pSmad, we observed greater increases in rhBMP-2 mediated signaling in the white matter than the grey matter. One plausible explanation could be the physical proximity of the white matter columns to the periphery of the spinal cord, and hence to the rhBMP-2 soaked sponges on the spine. Conversely, there was a higher baseline pSmad signaling in the gray matter of the uninjured cord, a region that is predominantly neuronal. Therefore, the post-injury activation of BMP

signaling in astrocytes and oligodendrocytes, which make up the dorsal column cell population, may have contributed to an immediate change in white matter pSmad counts. Furthermore, the most pronounced differences between pSmad activation in grey and white matter were observed at 7dpl. This finding correlated with the prominent increase of pSmad immunoreactivity in the meningeal fibroblasts invading the glial scar and the activated microglia/macrophages surrounding the lesion. Qualitatively, these cells appeared to localize within the white matter surrounding the lesion. Our results are in concordance with the work by Batchelor et al (Batchelor et al., 2008) who demonstrated that SCI generates a greater inflammatory response in the white matter with peak numbers of macrophages and microglia recruited around 7dpl. Thus, the largest increase in pSmad labeling correlates with the time when the highly BMP-responsive cell types infiltrate the white matter surrounding the lesion.

rhBMP-2 induced intrathecal signaling and permeability of the BSCB and meningeal barriers

As a secondary objective of this study we correlated the extent of rhBMP-2 induced pSmad activation with the BSCB and meningeal barrier permeability. In agreement with previous work, our results indicate an immediate disruption of the BSCB and meningeal barriers, which peaked at 7dpl (Whetstone et al., 2003). We established a highly significant correlation and a linear regression relationship between the protective barrier damage and intrathecal pSmad signaling. Furthermore, as the BSCB appeared to reform at 21dpl, pSmad immunoreactivity in the rhBMP-2 treated animals returned to pre-injury levels. Of note, however, is a persistent trend for increased pSmad signaling in

both the laminectomy only and 21dpl SCI groups treated with rhBMP-2. In both groups there was a respective 26% and 27% higher total density of pSmad positive cells, than in animals implanted with the control ACS. These differences, while not statistically significant, suggest that rhBMP-2 may penetrate through the intact meninges. This concept warrants further investigation as it has significant clinical implications.

The results from the first study have unequivocally demonstrated that exogenous rhBMP-2 activates signaling within the spinal cord; however, they did not provide data as to the long-term morphologic or functional effects of rhBMP-2.. Therefore, we performed another set of *in vivo* experiments that helped elucidate the acute and long-term effects of the exogenous protein on the morphologic changes within the spinal cord as well as on functional recovery.

Acute effects of implanting rhBMP-2 to the spine at the level of a SCI

Following the same injury protocol as described in the first study, SCI was induced via a dorsal hemisection at T9 to generate the worst-case scenario for exposing the spinal cord parenchyma to the exogenous protein. As surgical decompression is advocated within the first 8 to 24 hours after SCI clinically, we implanted collagen sponges with or without rhBMP-2 30 minutes after dorsal hemisection (deemed appropriate secondary to the differential metabolic rates between the animal model and humans) (Fehlings and Perrin, 2006).

Following a 7 day survival period (acute stage), we observed a pronounced increase in the macrophage/microglial staining in animals receiving rhBMP-2. This finding was in direct correlation with the original study, in which we observed a highly

significant BMP-specific signaling activation in macrophages/microglia at 7dpl. Current data further confirmed our earlier prediction that macrophage sensitivity to the exogenous rhBMP-2 may exacerbate intrathecal inflammation as both the invading macrophages and activated resident microglia are known mediators of post-SCI inflammatory response. In addition, these cell types have been shown to contribute to the secondary cell death through the release of free radicals, tumor necrosis factor α (TNF-α) and nitric oxide (NO) (Hausmann, 2003). Furthermore, a recent study by Hong and colleagues demonstrated that macrophage culture treatment with BMP-6 induces expression of pro-inflammatory nitric oxide synthase (iNOS) and TNF-α by the cells (Hong et al., 2008). Amino acid sequence similarity between BMP-6 and BMP-2 allows to hypothesize that rhBMP-2 could also stimulate production of the pro-inflammatory cytokines by these cells (Rueger, 2002). This concept requires further investigation as post-operative inflammation is one of the reported clinical complications associated with the "off-label" use of rhBMP-2. In a rat model of SCI, previous studies have established that the peak intrathecal inflammatory response falls on about the 7^{th} day post-injury (Batchelor et al., 2008). Thus the profound functional deterioration that we observed on the BBB scale and the base of support measurement at 7dpl could be in part attributed to the maximum inflammatory reaction triggered by rhBMP-2. This assumption is corroborated by the subsequent improvement in both parameters to the level of the control animals at 14dpl.

Concurrent with macrophage upregulation, spinal cords of animals treated with rhBMP-2 revealed increased labeling with GFAP, a well established marker of astrocyte reactivity and tissue gliosis. These data are again in concurrence with the results of the signaling study, which showed nuclear pSmad co-localization in the GFAP positive cells.

Following spinal cord trauma reactive astrocytes play a critical role in the formation of a glial scar and production of the inhibitory CSPGs that impede axonal regeneration through the lesion (Fitch and Silver, 2007). We believe that rhBMP-2 had a direct effect on astrocyte hypertrophy and GFAP expression as our separate *in vitro* studies using primary rat astrocyte cultures showed upregulation of GFAP mRNA following rhBMP-2 treatment. In addition, we observed a twofold increase in total CSPG immunoreactivity in areas surrounding the lesion of the rhBMP-2 rats compared to the controls. This finding correlated with the increased macrophage and astrocyte immunoreactivity as both of these cell types are known to produce inhibitory CSPGs. With regard to specific proteoglycans generated, we observed a strong trend for increased NG2 immunoreactivity in the experimental group, however there were no differences in neurocan immunostaining.

Meningeal fibroblasts invading the lesion form the core of the fibroglial scar, which constitutes a major physical barrier to axonal growth (Fawcett, 2006). In turn, they produce several extracellular matrix molecules including semaphorin 3 and NG2 that further contribute to the non-permissive qualities of the lesion core (Shearer et al., 2003). Earlier data revealed extensive pSmad co-staning with the invading meningeal fibroblasts in the rhBMP-2 treated rats. Results from the second study showed, that rhBMP-2 contributed to a marked increase in tissue fibrosis and ectopic scar formation.

Long-term effects of implanting rhBMP-2 to the spine at the level of a SCI

Similar trends in the composition of the fibroglial scar persisted at 6 weeks. Increased inflammation, GFAP immunoreactivity and NG2 deposition were evident on

the immunofluorescent analysis of the spinal cords from the rhBMP-2 group compared to the controls. These findings highlighted the long-term morphologic changes within the spinal cord following rhBMP-2 implantation to the spine. In addition, FDA submission data reports a twofold decrease in the amount of the recombinant protein at the site of implantation following a 10 day post-operative period in a rat. By 4 weeks after surgery local retention of rhBMP-2 in the ACS is altogether negligible (FDA, 2002). Therefore, morphologic changes within the spinal cord observed at 6 weeks post-SCI, persisted long after all of the protein has been cleared from the surgical site.

In our project, solid fusion masses were observed in a 100% of cases treated with rhBMP-2 as evidenced by detailed microCT analysis. Subsequent multiplanar CT data reconstructions showed no evidence of bone formation in the spinal canal in any of the cases. Comprehensive radiographic assessment allowed us to conclude that all morphologic and functional changes observed at the long-term follow up were secondary to the direct effect of rhBMP-2 rather than chronic compression of the spinal cord.

The effects of rhBMP-2 on axonal sprouting and regeneration

Anterograde labeling of the descending fibers comprising the CST tract was performed in our study to analyze whether rhBMP-2 had an effect on axonal regeneration. Dorsal hemisection SCI results in the complete transection of the descending fibers of the CST tract; therefore, axons labeled with the fluorescent dye distal to the lesion are the product of spontaneous regeneration. Results from this experiment revealed dense areas of axonal sprouting within the lesion core in both groups, with a trend for increased number of axons in the rhBMP-2 group. The direct

effects of rhBMP-2 on neuronal growth are not fully understood. Several previous studies presented conflicting data on this matter. Matsuura and colleagues inhibited endogenous BMP signaling within the spinal cord by intrathecally infusing BMP-specific antagonist, noggin, and observed improved functional recovery and CST axon growth following an SCI (Matsuura et al., 2008). In contrast, Zou et al (Zou et al., 2009) has recently published that axotomy induced Smad-1 upregulation (BMP-specific downstream transcription factor) increases axonal growth in the adult sensory neurons. In the current project, CST axonal sprouting in the BMP group was moderately increased rostral to the lesion; however, axonal numbers distal to the injury were markedly reduced relative to the control SCI group. This observation requires further study as uncontrolled axonal sprouting could result in improper synapse formation in the dorsal horn and post-injury onset of neuropathic pain. In the rat, afferent sensory axons synapse on spinal interneurons in laminae 1, 2, whereas descending CST fibers terminate on the respective lower motor neurons, which are found in laminae 3-6 (Hodgetts et al., 2009). Improper signaling could facilitate progression of the chronic pain syndrome, a frequent post-SCI complication reported clinically and in animal models (Sjolund, 2002; Hulsebosch, 2005; Nesic et al., 2005). A profound manifestation of neuropathic pain in the spinal cord injured rats is the development of autophagia. Interestingly, in our study this phenomenon was noted in 56% of rhBMP-2 treated rats versus only 35% of the control animals. Whether rhBMP-2 elicited this response through increased sprouting or other mechanisms remains to be determined in future studies; however, macrophages and reactive glia are known modulators of mechanical allodynia (Stuesse et al., 2001; Ji and Suter, 2007). Thus rhBMP-2 could have an indirect effect on post-injury allodynia

through upregulation of macrophage infiltration and astrocyte reactivity. However, rhBMP-2 could also have a direct effect on the expression of various neuropeptides regulating pain in the dorsal horn and dorsal root ganglia. Clinically, post-operative radiculitis is one of the recently reported post-operative complications in patients undergoing transforaminal lumbar interbody fusion (TLIF) with rhBMP-2 (Rihn et al., 2009). Therefore, prior to drawing further conclusions, additional studies are necessary to fully elucidate the exact role and mechanism of rhBMP-2 on the different sensory and motor neurons of the spinal cord, as well as its modulation of pain.

Functional changes associated with rhBMP-2 implantation to the spine

Functionally, we did observe significant differences between the control and rhBMP-2 treated animals at the 6 week time point, which were manifested in changes of the hind paw exorotation when compared to the baseline reading. Unlike in humans, corticospinal tract (CST) lesion in a rat does not preclude from spontaneously regaining function of the hind limbs, including locomotion (Hodgetts et al., 2009). Instead, over the long-term, CST lesions are associated with the loss of fine motor skills, including paw position control (Muir and Whishaw, 1999). Therefore, rhBMP-2 had a significant detrimental effect on functional performance that was appropriately assessed by the digital CatWalk system. BBB testing, although repeatedly validated for measuring functional recovery following a contusion SCI, was not sufficiently sensitive to delineate functional changes in our study. Nevertheless, we did observe significant deterioration of functional performance on the BBB rating at one week after injury that corresponded to the peak inflammatory response elicited by rhBMP-2.

In conclusion, the results from our studies have demonstrated for the first time that rhBMP-2 used in spinal arthrodesis in the vicinity of a SCI, elicits a direct functional response within the spinal cord parenchyma. The extent of direct intrathecal signaling is dependent on the integrity of the BSCB and meningeal barriers; however, further studies must be conducted to evaluate rhBMP-2 penetration through the intact meninges. In the presence of a SCI, rhBMP-2 implantation to the spine generates extensive negative effects on the course of spinal cord pathology, which are manifested through increased intrathecal inflammation, gliosis, fibrous scarring and deposition of inhibitory CSPGs. Ultimately, this response contributes to a decrease in functional recovery at the chronic stage. Our findings, although obtained in a rat model of dorsal hemisection SCI, suggest that the use of rhBMP-2 may not be the optimal choice in surgical management of patients with spinal cord pathology or significant dural deficiencies.

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