The Role of TIMP-1 after Spinal Cord Injury

By

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ABSTRACT

Spinal cord injury (SCI) is a traumatic nerve injury that typically results in permanent loss of motor function and sensory function. Currently, there are no cures for SCI. Following SCI in rodents, several inflammatory cytokines are upregulated and cause secondary damage. Tissue inhibitor of metalloproteinases (TIMP-1) is upregulated post-injury and promotes astrocyte proliferation among many functions. If further upregulated after SCI, TIMP-1 could attract astrocytes to the compromised blood vessels and attenuate the immune response, lessening the secondary damage. The objective of this study was to quantify the inflammatory cytokines that are present 24 hours after SCI when (1) TIMP-1 is injected via tail vein injection three hours post-SCI and (2) TIMP-1 bound to mineral-coated microparticles (MCMs) are injected directly into the spinal cord six hours post-SCI. It was found that inflammatory chemokines and cytokines levels are not significantly different between injured control and TIMP-1 treatment rats.

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INTRODUCTION

Spinal cord injury (SCI) paralyzes nearly 12,500 people every year in North America[1], and an estimated 262,000 people were living with SCI in 2016[2]. Currently, there are no cures for SCI, and patients who suffer from SCI typically have chronic disability and experience motor and sensory functioning loss[1,3].

Primary damage of the spinal cord is characterized by death of glial cells and damaged vasculature and is due to the initial mechanical injury[4]. When the glia and neurons of the spinal cord are injured, a cascade occurs which increases inflammation and neural degradation. This causes secondary damage to the spinal cord and prevents functional recovery. Existing literature indicates that interleukin 1α (IL-1α), interleukin 1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) are among the inflammatory cytokines that are upregulated between 2 hours and 14 days post-SCI in rodent models[1]. Various cytokines and chemokines are secreted by astrocytes, macrophages, and microglia to guide innate and adaptive immune cells to the site of injury. Anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13, can downregulate these inflammatory cytokines. However, past literature indicates that anti-inflammatory cytokines are less concentrated around the injury site following SCI. Thus, the majority of cytokines present are proinflammatory, leading to an increased amount of inflammation[1].

During this secondary cascade, TIMP-1 plays an important role in CNS repair by inhibiting matrix metalloproteinases (MMPs). MMPs are zinc endopeptidases that are secreted by leukocytes, and they activate proinflammatory cytokines and regulate extracellular matrix (ECM) turnover[5, 6]. In response to these proinflammatory cytokines, TIMP-1 is secreted by astrocytes and inhibits matrix metalloproteinase-9 (MMP-9). TIMP-1 aids in formation of glial scars, builds the ECM, and promotes astrocyte proliferation[7,8]. In a previous study, rats underwent spinal cord injury, and the cytokine/chemokine profile was quantified (**Fig. 1**). It was found that endogenous TIMP-1 is upregulated post-injury to the spinal cord and has a peak concentration occurring at 24 hours post-SCI in rodents (**Fig. 1A**). In addition, MCP-1 and MIP-1α are

chemokines that recruit inflammatory cells to the site of injury and are typically also upregulated post-injury (**Fig. 1B, 1C**).

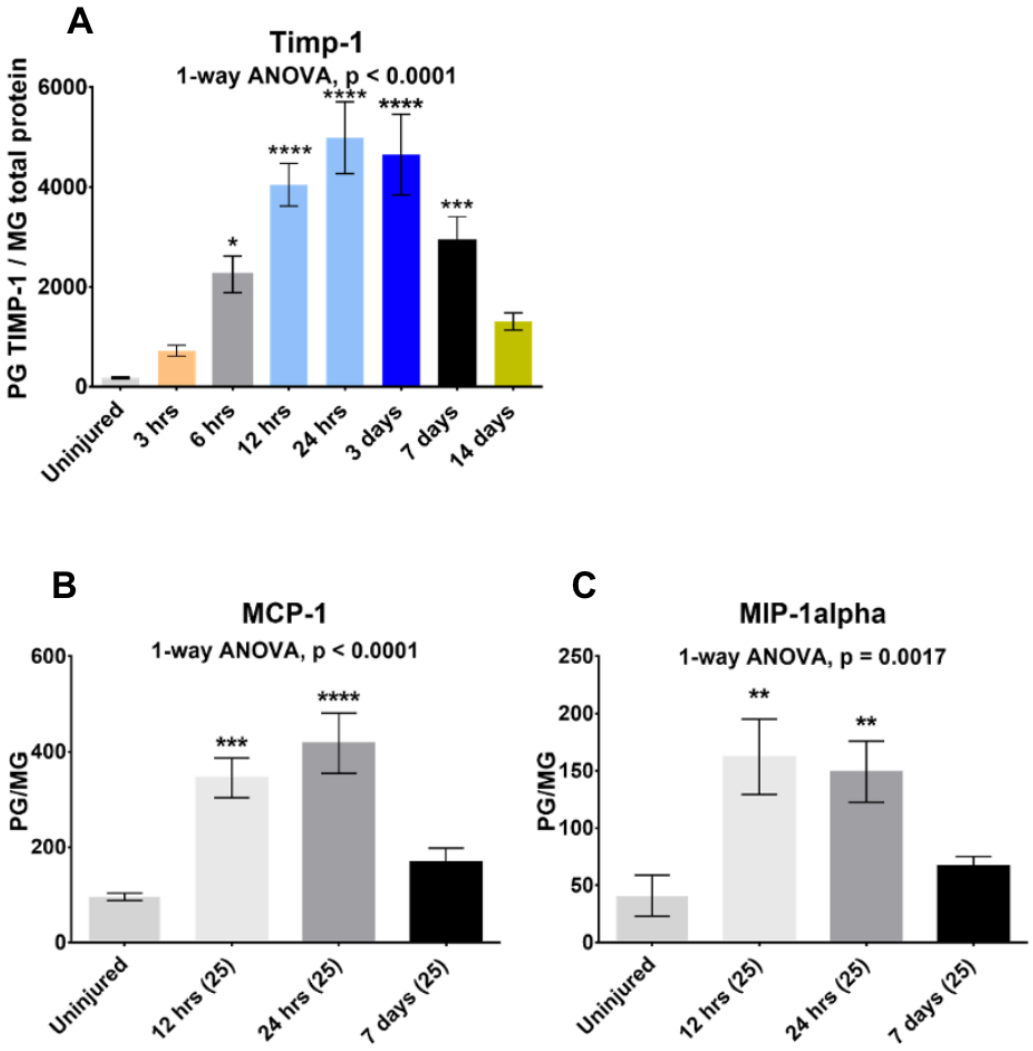

Figure 1. Levels of TIMP-1, MCP-1, and MIP-1α at different timepoints following SCI. After spinal cord contusion in a rat, spinal cords were harvested and homogenized at varying times post-injury, and the levels of these factors were measured. One-way ANOVA was used followed by Dunnett's post-hoc analysis to compare to the uninjured controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **A.** TIMP-1 is upregulated after SCI, reaching a peak concentration at 24 hours post-injury. **B, C.** MCP-1 and MIP-1α are upregulated 12 to 24 hours post-injury and attract immune cells to the site of injury.

TIMP-1 can function via two distinct pathways that benefit the repair mechanisms: MMP-dependent and MMP-independent pathways (**Fig. 2**). When MMP is present, TIMP-1 binds to either free MMP or membrane-bound MMP. MMP-dependent functions of TIMP-1 include ECM remodeling, preservation of the blood-spinal cord barrier, and less neutrophil migration to the site of injury[7].

In an MMP-independent manner, TIMPs have cytokine-like functions in which they bind to specific TIMP surface receptors[6]. TIMP-1 can bind to either proMMP-9 in an MMP-dependent manner or CD63 in an MMP-independent manner, and both compete to bind TIMP-1. CD63 is the receptor that suppresses apoptosis in downstream cascades. However, TIMP-1 has a higher affinity for proMMP-9 compared to CD63. Thus, when proMMP-9 is elevated post-SCI, there is less free TIMP-1 available to bind with CD63, and therefore, the MMP dependent pathways are favored[6]. Using tissue inhibitor of metalloproteinases (TIMP-1) to alter the timeline of astrocyte proliferation may be a potential treatment for spinal cord injury.

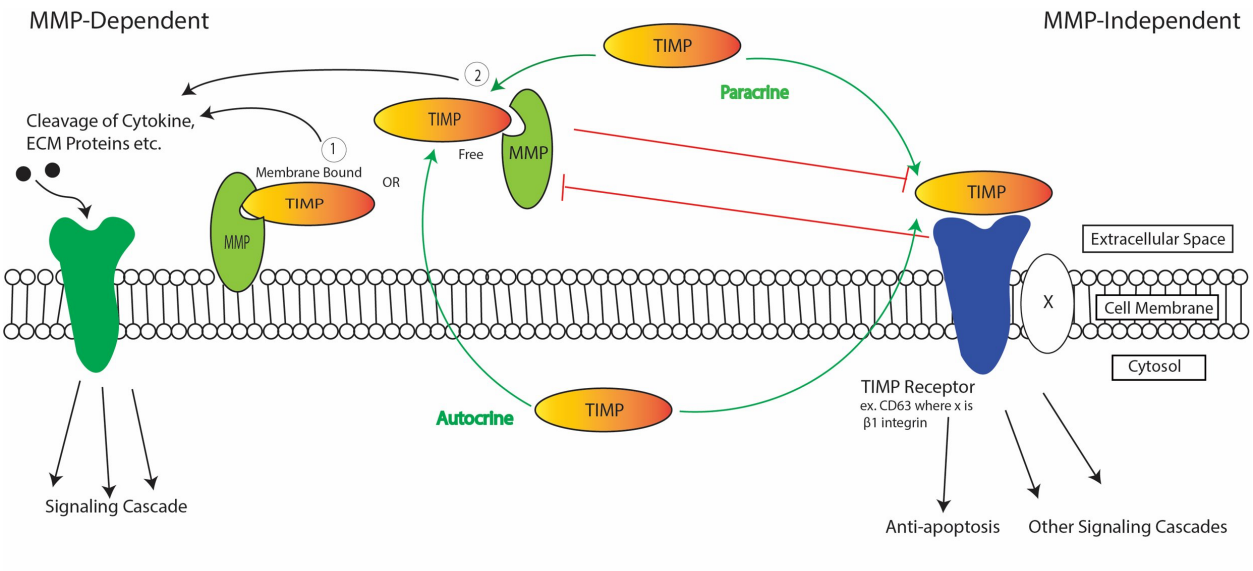

Figure 2. MMP-dependent and MMP-independent functions of TIMP-1. One type of MMP-independent TIMP-1 signaling is mediated through CD63/β1-integrin/Akt, as depicted by "x." Adapted from Ries 2014[6].

In this study, the downstream effects of TIMP-1 on the level of inflammation after SCI were examined. This was done by upregulating TIMP-1 around the damaged blood vessels in the spinal cord three hours post-SCI, which is earlier than when the endogenous TIMP-1 concentration peaks. Typically, endogenous TIMP-1 has a peak concentration occurring at 24 hours post-SCI and is minimally present after three hours (**Fig. 1A**). It was hypothesized that after SCI, an injection of TIMP-1 will attract astrocytes to the compromised blood vessels to encase and attenuate the infiltrating immune cells and thus reduce inflammatory cytokines. Levels of chemoattractants MCP-1 and MIP-1α also will likely be decreased 24 hours post-SCI after TIMP-1 injection. To accomplish this, either an intravenous (IV) treatment of TIMP-1 or an intra-spinal cord treatment of TIMP-1 bound to mineral-coated microparticles (MCMs) was given three hours or six hours after a rat spinal cord contusion, respectively. MCMs allow for a sustained release of TIMP-1, where TIMP-1 can bind the charged surface of the MCM (**Fig 3**). The levels of inflammatory cytokines and chemokines were measured after 24 hours.

Currently, adjusting the timeline of astrocyte proliferation using TIMP-1 has not yet been used as treatment for spinal cord injury. It was found in this study that inflammatory chemokines and cytokines levels are not significantly different between injured control and TIMP treatment rats.

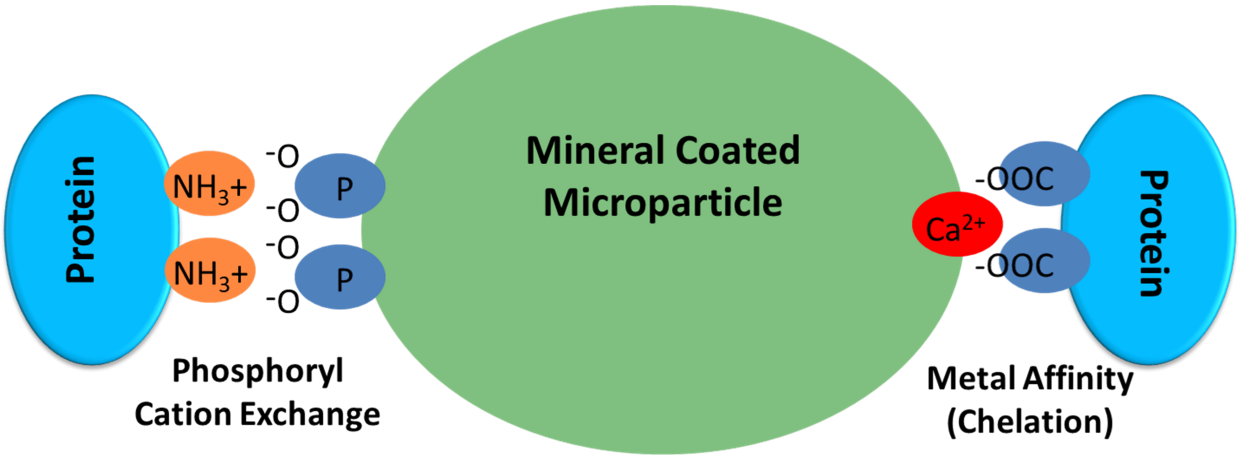

Figure 3. Schematic diagram of mineral coated microparticles. Protein binding via electrostatic interactions between protein side chains and the charged surface on mineral coating allow for sustained protein release.

METHODS

Four experimental groups were created: (1) uninjured controls, (2) injured controls without TIMP-1 treatment, (3) injured subjects with IV TIMP-1 treatment, and (4) injured subjects with TIMP-1 and MCMs injected into the spinal cord.

32 Sprague Dawley rats underwent a laminectomy at the T10 and T11 vertebrae to expose a portion of the spinal cord. The spinal cords of the rats in the injured groups were then contused by dropping a 10g weight from 25mm above the spinal cord (**Fig. 4**). For group 3, the rats were injected with 160ng of TIMP-1 mixed in 500uL of rat plasma via tail vein injection three hours post-SCI. This was calculated in order to reach a concentration of 10ng/mL TIMP-1 in a rat that contains approximately 16mL of blood[8]. In group 4, the rats received 3µL MCM (approximately 80ng) saturated with TIMP-1 injected directly into the spinal cord six hours post-SCI. The rat plasma was purchased from Innovative Research, and TIMP-1 was purchased from R&D.

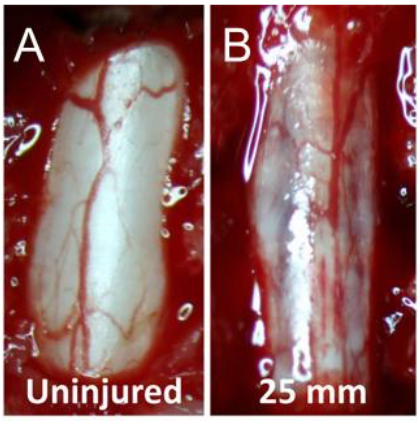

Figure 4. Representative spinal cord segments that A. are uninjured, and B. have been contused by dropping a 10g weight directly onto the spinal cord from 25mm above. Spinal cords were exposed by performing a T10-T11 laminectomy.

To determine group sizes for measuring cytokine levels in the spinal cord, a power of analysis was conducted with 80% power and a significance level (α) of 0.05 to detect a difference in means of at least 20% (Student's t-test two-tailed, StatMate). From our previous multiplex assays, we had a sample

standard deviation of 12%. Thus, for measuring the level of cytokines, a sample size of 7 in each group has 80% power to detect a difference between means of 20%. Thus, 8 rats per group (4 female rats and 4 male rats) were used for a total of 32 rats.

To measure cytokine levels, the rats were euthanized 24 hours post-SCI. 7 mm of spinal cord at the epicenter of the injury were harvested and weighed, placed in lysate buffer, homogenized, and sonicated. The supernatants were frozen at -80ºC until later analysis. A BCA protein assay was run, and all samples were diluted to 5mg total protein per mL. The samples were then analyzed with a 27-plex cytokine/chemokine array (Eve Technologies) to measure the levels of Eotaxin, EGF, Fractalkine, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, GRO/KC, TNFα, G-CSF, GM-CSF, MCP-1, Leptin, LIX, MIP-1α, MIP-2, RANTES, and VEGF.

All results were analyzed using Prism GraphPad Software. One-way ANOVA was used to analyze cytokine levels. Following up the ANOVA test, Tukey's post-hoc analysis was used to test between groups or Dunnett's post-hoc analysis was used to compare treatments to the controls as they have greater power to detect differences between groups. Differences are considered significant at a probability level of 95% ($p<0.05$).

RESULTS

In order to determine whether IV TIMP-1 (IV-TIMP-1) injections or TIMP-1 bound to MCMs (MCM+TIMP-1) injected directly into the spinal cord reduces inflammation locally into the injury site of the spinal cord, cytokine/chemokine assays were performed on the spinal cord homogenates. TIMP-1 levels 24 hours post-SCI were not significantly different amongst the injured controls, IV-TIMP-1 rats, and MCM+TIMP-1 rats. Uninjured rats had virtually no TIMP-1 present in the spinal cord homogenates (**Fig. 1A**). Correspondingly, uninjured rats had a significantly higher level of MMP-9 in the spinal cord compared

to all the injured groups, but there was no significant difference between the injured control and the TIMP-1 groups (**Fig. 1B**).

MCP-1 and MIP-1α were at very low concentrations in uninjured rats and elevated in all injured groups with no significance difference between them. IV-TIMP-1 rats had slightly higher, albeit statistically insignificant, MCP-1 concentration (**Fig. 1C, Fig. 1D**).

IV-TIMP-1 rats contained a significantly higher concentration of leptin within the spinal cord compared to uninjured rats, nearly 2.5-fold, with $p<0.0001$. MCM+TIMP-1 rats also had a significantly elevated level of leptin compared to uninjured rats with $p<0.05$. Injured and uninjured rats contained statistically similar concentrations of leptin (**Fig. 1E**).

G-CSF levels were statistically different in injured controls and MCM+TIMP-1 rats compared to uninjured controls while there is no statistical difference between IV-TIMP-1 rats and uninjured rats (**Fig. 1F**). Similarly, there is no statistical difference in fractalkine levels between IV-TIMP-1 rats and uninjured rats, but injured controls and MCM+TIMP-1 rats have significantly lower levels compared to uninjured controls (**Fig. 1G**). There were also no significant differences in IL-1β levels and levels of all other cytokines and chemokines measured between groups.

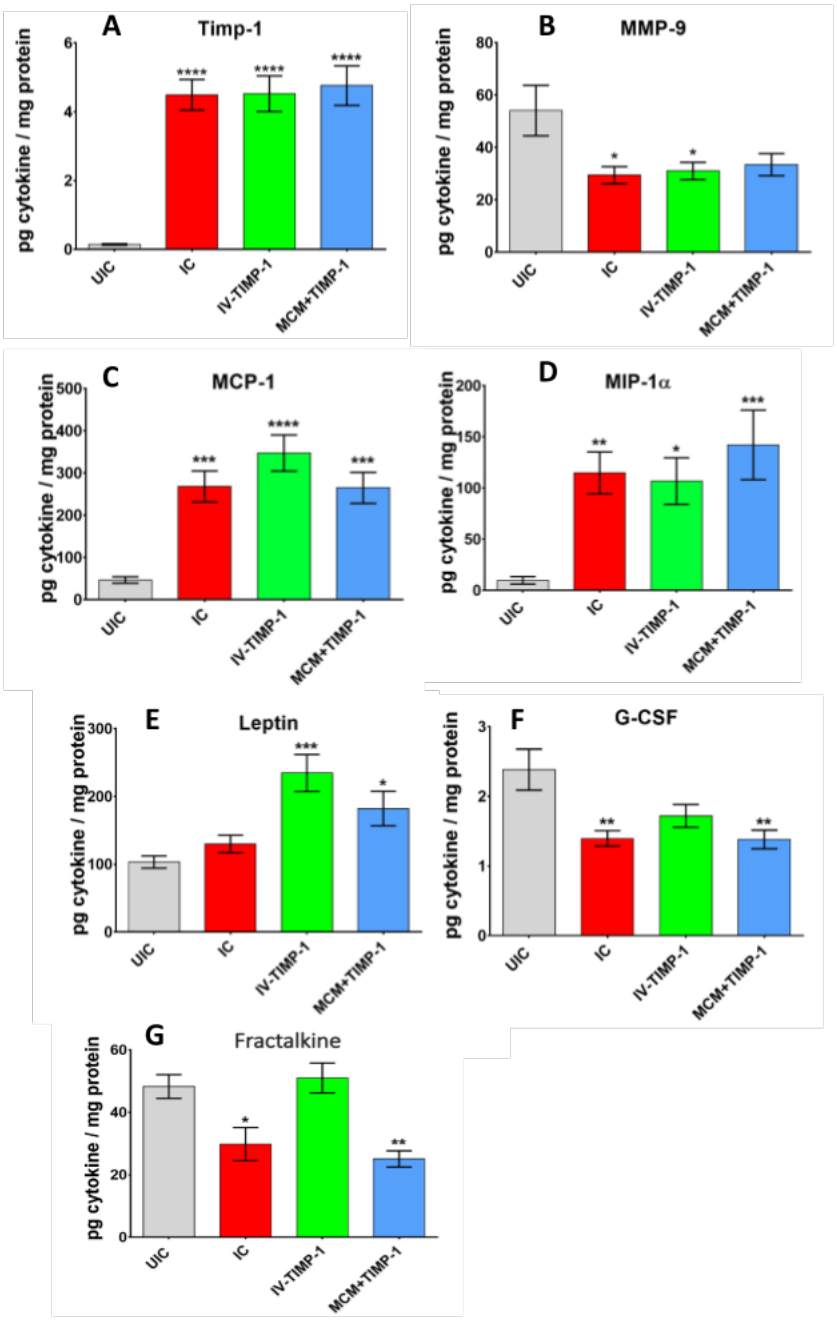

Figure 5. Select cytokine/chemokine levels present in the spinal cord 24 hours post-SCI. UIC = uninjured control; IC = injured control; IV-TIMP-1 = intravenous TIMP-1 injection three hours post-SCI; MCM+TIMP-1 = injection of TIMP-1 bound to MCMs six hours post-SCI. Analyzed using one-way ANOVA and Tukey's post-hoc analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to UIC.

DISCUSSION

In order to determine the effect of TIMP-1 treatments on inflammation present in the spinal cord after SCI, a cytokine and chemokine assay was performed. Injecting TIMP-1 either intravenously or bound to MCMs did not have an impact on the TIMP-1 levels present in the spinal cord 24 hours after SCI. It is possible that the endogenous TIMP-1 upregulation at 24 hours after injury is significantly more intense and was not affected by the injected TIMP-1 treatments. This explains the lack of many significant differences in cytokine and chemokine levels between injured control rats and TIMP treatment rats.

MMP-9 levels corresponded with the TIMP-1 levels as TIMP-1 inhibits MMP-9 directly. Thus, it is reasonable to assume that MMP-9 levels are high in uninjured controls, where TIMP-1 levels are low, and low in all injured groups, where TIMP-1 levels are high. This is what is seen in the results, where MMP-9 levels are elevated and have no significant differences between all injured groups. MCP-1 and MIP-1α are proinflammatory cytokines, and they are both elevated in all injured groups as well. Because MMP-9, MCP-1, and MIP-1α are all inflammatory in nature, it indicates that there are not many significant differences in inflammation between injured controls and TIMP-1 treatment rats.

However, there is evidence that the TIMP-1 treatments had some impact on the cytokine/chemokine profile of the rats' spinal cords. Leptin aids in neuroprotection and cell survival, and it is anti-inflammatory in nature[9]. Both TIMP-1 treatments have significantly elevated leptin levels when compared to both the injured and uninjured controls, indicating that though the proinflammatory cytokines levels are not lower in TIMP-1 treatment rats, there may be some increased anti-inflammatory factors that can aid in recovery after SCI.

G-CSF can decrease inflammatory cytokines IL-1β and TGFα levels after SCI and suppress apoptosis of neurons[10]. G-CSF is elevated in uninjured controls and depressed in injured controls. However, IV-TIMP-1 treatment was able to regenerate the G-CSF levels in the spinal cord such that there is no significant

difference between the levels in uninjured rats and IV-TIMP-1 rats, which is a promising result. For fractalkine, there is evidence that it may be inflammatory in nature, but the pathways are not yet known[11]. However, IV-TIMP-1 has comparable levels of fractalkine to the uninjured controls while the injured controls and MCM+TIMP-1 rats could not regenerate this level of fractalkine. This indicates that IV-TIMP-1 treatment may be working to bring the cytokine/chemokine profile back to the profile of a rats' uninjured state, but more studies are needed to confirm this association.

Overall, inflammatory cytokine and chemokines levels are not significantly different between injured control and TIMP treatment rats. This can be due to the timeline of measuring cytokine and chemokine levels 24 hours after injury. It would be useful to measure cytokine and chemokine levels 2-3 hours after treatment. In addition, since TIMP-1 was upregulated earlier than the endogenous TIMP-1, it may still have long-term effects on lesion size and functional recovery that may not have been captured by this study. It would be beneficial to measure the difference in lesion size and glial scars on the spinal cords of TIMP-1 treatment rats compared to control rats with a longer timeline. Glial scars are typically established around 28 days after SCI, so the impact of TIMP-1 using functional analysis techniques and lesion size measurements may be different than the results indicated in this study.

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