Localized and Sustained Delivery of Fibroblast Growth Factor-2 from a Nanoparticle-Hydrogel Composite for Treatment of Spinal Cord Injury

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FGF2 achieved higher blood vessel density in the dorsal horns 28 days post-injury, due to either greater angiogenesis near the epicenter of the injury or vasoprotection acutely after spinal cord injury. Importantly, delivery of FGF2 from composite HAMC/PLGA/FGF2 did not produce proliferative lesions that had been previously reported for FGF2 delivered locally using a minipump/catheter. These results suggest that localized and sustained delivery with composite HAMC/PLGA/FGF2 is an excellent system to deliver biomolecules directly to the spinal cord, thereby circumventing the blood spinal cord barrier and avoiding systemic side effects.

Key Words
Growth factors · Traumatic spinal cord injury · Therapeutic approaches · Central nervous system injury

Abstract
After traumatic spinal cord injury, grossly injured blood vessels leak blood and fluid into the parenchyma, leading to a large cystic cavity. Fibroblast growth factor-2 (FGF2) can reduce immediate vasoconstriction of vessels in the tissue surrounding the primary injury and promote angiogenesis. A localized delivery system would both achieve restricted delivery of FGF2 to the spinal cord and limit possible systemic effects such as mitogenesis. To enhance the endogenous angiogenic response after spinal cord injury, FGF2 was encapsulated in poly(lactide-co-glycolide) (PLGA) nanoparticles which were embedded in a biopolymer blend of hyaluronan and methylcellulose (HAMC) and then injected into the intrathecal space. Treatment began immediately after a 26 g clip compression spinal cord injury in rats and consisted of intrathecal delivery of FGF2 from the HAMC/PLGA/FGF2 composite. Control animals received intrathecal HAMC loaded with blank nanoparticles, intrathecal HAMC alone or intrathecal artificial cerebrospinal fluid alone. Sustained and localized delivery of FGF2 from composite HAMC/PLGA/FGF2 achieved higher blood vessel density in the dorsal horns 28 days post-injury, due to either greater angiogenesis near the epicenter of the injury or vasoprotection acutely after spinal cord injury. Importantly, delivery of FGF2 from composite HAMC/PLGA/FGF2 did not produce proliferative lesions that had been previously reported for FGF2 delivered locally using a minipump/catheter. These results suggest that localized and sustained delivery with composite HAMC/PLGA/FGF2 is an excellent system to deliver biomolecules directly to the spinal cord, thereby circumventing the blood spinal cord barrier and avoiding systemic side effects.

Abbreviations used in this paper
aCSF artificial cerebrospinal fluid
BBB Basso, Beattie and Bresnahan
FGF2 fibroblast growth factor-2
HA hyaluronan
HAMC hyaluronan and methylcellulose
HE hematoxylin-eosin
LFB Luxol fast blue
MC methylcellulose
PLGA poly(lactide-co-glycolide)
SCI spinal cord injury
**Introduction**

Traumatic spinal cord injury (SCI) is a devastating condition that is characterized by extensive tissue degeneration and severe loss of function. The primary mechanical injury causes immediate hemorrhage and ischemia, in turn causing hypoxia, inflammation, edema and ultimately widespread cell death. This secondary injury extends the tissue damage of the primary injury and may continue for weeks after the initial trauma, in part due to a decrease in blood flow. Clinical treatment to counteract the secondary injury is very limited and may involve intravenous administration of the steroid methylprednisolone; however, the efficacy of this treatment has been widely debated [Fehlings, 2001]. Several clinical trials have been completed with pharmacological agents, as reviewed in Tator [2006], Fehlings [2001] and Hawryluk et al. [2008]; yet no treatment has shown sufficient benefit to be established as a standard clinical practice. Improving blood flow to the injured tissue could have a profound benefit by limiting degeneration resulting from ischemia.

Blood flow in the spinal cord is impacted acutely after injury when vasoconstriction occurs due to factors that are released from immediate cell death from the primary injury as well as through the process of angiogenesis where existing blood vessels branch and extend to increase vasculature. After SCI, the vasculature increases [Loy et al., 2002] and spontaneously remodels for weeks after the primary injury [Popovich et al., 1996]. Fibroblast growth factor-2 (FGF2) has been shown to promote angiogenesis [Shing et al., 1985; Montesano et al., 1986; Relf et al., 1997], be neuroprotective [Nozaki et al., 1993; Lee et al., 1999] and cause vasodilation [Cuevas et al., 1991; Ziche and Morbidelli, 2000]. In addition, FGF2 may reduce permeability of the blood-spinal cord barrier [Reuss et al., 2003; Kang et al., 2010a]. In vivo delivery of FGF2 from an osmotic minipump demonstrated enhanced functional recovery in spinal cord-injured rats [Rabchevsky et al., 1999, 2000] and FGF2 has been tested clinically for the treatment of stroke [Bogousslavsky et al., 2002]. Thus, we hypothesized that FGF2 has the potential to reduce the ischemic injury and promote angiogenesis following SCI.

Although systemic delivery is the most common clinical practice for drug administration, this route is not suitable for FGF2 because it does not cross the blood-spinal cord barrier [Relf et al., 1997] and its mitogenic potential may lead to malignant tumors [Relf et al., 1997]. Localized drug delivery to the intrathecal space has been used to circumvent these limitations, either as a bolus injection or with implantable catheters and minipump devices. However, bolus delivery is transient and implantable devices may cause infection and compression of the cord [Jones and Tuszynski, 2001]. To overcome the shortcomings of these techniques, we developed a minimally invasive, intrathecal drug delivery system that is effective and safe for local delivery of therapeutic agents to the site of injury [Gupta et al., 2006; Kang et al., 2009]. This localized delivery system is composed of a biopolymer blend of hyaluronan and methylcellulose (HAMC), and has been used to locally deliver FGF2 into the injured spinal cord from 30 min to 6 h [Kang et al., 2010b]. This short-term delivery of FGF2 resulted in a moderate improvement in spinal cord blood flow and a decrease in the permeability of the blood-spinal cord barrier [Kang et al., 2010a].

To extend the delivery period beyond 6 h to a time frame relevant to angiogenesis, FGF2 was encapsulated in poly(lactide-co-glycolide) (PLGA) nanoparticles. These FGF2-loaded nanoparticles were dispersed in a formulation of HAMC optimized for long-term drug delivery based on in vitro swelling, degradation and drug release [Baumann et al., 2009]. The safety and biocompatibility of this composite HAMC/PLGA was established when injected in the intrathecal space [Baumann et al., 2010]. Here we investigate the tissue benefit of sustained release of FGF2 from HAMC/PLGA/FGF2 nanoparticle composite and, in so doing, test for the first time a sustained release, minimally invasive delivery system to the injured spinal cord. To limit vasoconstriction, which occurs immediately after injury and leads to tissue degeneration, soluble FGF2 was also dispersed within the hydrogel which has previously been shown to diffuse from the gel into spinal cord tissue between 30 min and 6 h post-injury [Kang et al., 2010]. The angiogenic response and tissue recovery resulting from local delivery of FGF2 from the HAMC/PLGA/FGF2 composite was compared to that of controls of blank HAMC/PLGA, HAMC alone and artificial cerebrospinal fluid (aCSF) alone.

**Materials and Methods**

Recombinant human FGF2 was purchased from Biovision (Mountain View, Calif., USA). Sodium hyaluronate of 2,600 kg/mol was purchased from Lifecore (Chaska, Minn., USA). Methylcellulose of 300 kg/mol was purchased from Shin-Etsu (Tokyo, Japan). Poly(D,L-lactide-co-glycolide) 50:50, of inherent viscosity 0.15–0.25 dl/g was purchased from Sigma-Aldrich. Poly(vinyl alcohol), 6 kg/mol and 80 mol% hydrolyzed, was purchased from Polysciences Inc. (Warrington, Pa., USA). HPLC-grade dichloromethane was supplied by Caledon Labs (Georgetown, Calif., USA).
After a laminectomy, the dura was punctured with a closing force of 26 g, as previously described [Rivlin and Tator, 1978].

Preparation of FG2-Loaded PLGA Nanoparticles

FGF2-loaded nanoparticles were prepared from a water-oil-water double emulsion, with an inner aqueous phase of 300 µl containing 4.17 mg/ml protein and 20 mg/ml heparin in aCSF, an organic phase of 2.7 ml, 50 mg/ml PLGA (0.15–0.25 dl/g) and 0.5 mg/ml Pluronic F-127 in dichloromethane, and an outer aqueous phase of 9 ml, 25 mg/ml poly(vinyl alcohol). The primary emulsion was created by sonicating for 10 min over ice. The secondary emulsion was formed by addition of the outer aqueous phase and sonication for a further 10 min over ice. The double emulsion was then added to 111 ml of a 25 mg/ml poly(vinyl alcohol) solution and stirred for 20 h at room temperature. Protein-loaded PLGA nanoparticles were isolated and washed 4 times by ultracentrifugation, lyophilized and stored at −20 °C prior to use.

Preparation of HAMC and HAMC Composite Hydrogels

Physical hydrogel blends of hyaluronan (HA) and methylcellulose (MC) were prepared in aCSF at 1 wt% 2,600 kg/mol HA and 3 wt% 300 kg/mol MC as previously reported (1:3 HMW HAMC [Baumann et al., 2009]). Briefly, sterile MC and HA were sequentially mechanically dispersed in aCSF and allowed to dissolve at 4 °C. A slurry of PLGA nanoparticles in aCSF was added to a concentrated HAMC solution to produce a composite hydrogel of 1 wt% HA, 3 wt% MC and 10 wt% PLGA. HAMC containing FG2-loaded nanoparticles was prepared similarly and also contained 10 µg/ml of soluble FG2 with a 1:20 w/w ratio of FG2:heparin in the gel phase. The nomenclature is hereafter abridged to HAMC/PLGA for non-FG2-containing hydrogel, and HAMC/PLGA/FG2 for HAMC loaded with soluble FG2 and FG2 nanoparticles.

In vitro Release of FG2 from HAMC/PLGA/FG2

A 100-µl aliquot of the HAMC/PLGA/FG2 mixture was injected into the bottom of a Eppendorf tubes containing 900 µl of aCSF at 37°C, approximating the ratio of HAMC to CSF that is expected in vivo by injection into the intrathecal space of a rat. These samples were incubated at 37°C on an orbital shaker and aCSF was fully removed and replaced with fresh aCSF at t = 1, 3, 5, 8, 14 and 18 days. A sandwich ELISA assay (R&D Systems, human FGF2 basic Duoset) was used to determine the concentration of FGF2 in the aCSF that was removed at each time point (n = 4).

In vivo Surgical Procedures

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals developed by the Canadian Council on Animal Care and approved by the Animal Care Committee at the Research Institute of the University Health Network. Forty female Sprague-Dawley rats (200–250 g; Charles River, Montreal, Que., Canada) were anesthetized by inhalation of halothane and a laminectomy was performed at the T1–T2 vertebral level. All animals sustained a moderate compressive SCI at T2 using a modified aneurysm clip calibrated to a closing force of 26 g for 60 s, as previously described [Rivlin and Tator, 1978]. After a laminectomy, the dura was punctured with a beveled 30-gauge needle at T2, and then a 30-gauge blunt-tipped needle was inserted into the intrathecal space. Animals were divided into 4 groups (n = 10/group) and each animal received 10 µl of either: (1) composite HAMC/PLGA/FG2; (2) composite HAMC/PLGA; (3) HAMC, or (4) aCSF, the buffer in which HA and MC were dissolved. All materials were maintained at 37°C prior to injection. Following injection, the overlying muscles and fascia were sutured closed and the rats were ventilated with pure oxygen and placed under a heat lamp for recovery. Buprenorphine was administered every 12 h for 3 days post-surgery for pain management. Motor function was assessed weekly as described below and sacrificed 28 days after surgery. Intracardiac perfusion with 4% paraformaldehyde was performed under terminal anesthesia with sodium pentobarbital. A 20-mm segment of the spinal cord encompassing the injury site was harvested from each animal, dehydrated in 30% sucrose, and stored at −80°C until processing. Cords were then cut with a cryostat as indicated below.

Histology and Immunohistochemistry

Four cords from each group were sectioned parasagittally at 20 µm thickness on a cryostat (CM 3050 S; Leica Microsystems, Wetzlar, Germany). Every 10th section in the clip-compression was stained with Luxol Fast Blue (LFB), and counter-stained with hematoxylin-eosin (HE). Images were captured by bright-field microscopy (Scanscope XT; Aperio Technologies, Vista, Calif., USA) at 20× objective magnification, examined for general tissue morphology, and the cavity area quantified with an image analysis system for all sections (Image J; Wayne Rasband, Bethesda, Md., USA). Cavity volume was calculated by multiplying the area per section by thickness per section and weighted by the sampling frequency.

Four cords from each group were cross-sectioned at 20 µm to observe cellular expression in the dorsal and ventral horns of the spinal cord. Fluorescent microscopy was used to assess co-staining of NeuN for neurons and SMI-71 for mature blood vessels at 20× objective magnification (BX-61; Olympus, Tokyo, Japan). The sections were taken at ±300, 600, 900 and 1,200 µm on each side of the injury epicenter. Neurons were identified by the NeuN TRITC conjugate staining and blood vessels were identified by SMI-71 FITC conjugate staining in microscopic images. Both neurons and blood vessels were counted in the left and right dorsal horns in a single field of view encompassing the entire dorsal horn, corresponding to 134.4 mm². Data for each respective stain was pooled for the right and left dorsal horns and are reported in counts/mm².

Functional Assessment

To determine the behavioral effects, open-field locomotor function was assessed weekly using the Basso, Beattie and Bresnahan (BBB) scoring method [Basso et al., 1995] for 4 weeks. Each hind limb was ranked by two blinded observers and concurrently videotaped.

Statistical Analysis

All statistics were performed using two factorial ANOVA followed by Tukey’s post hoc test. Counts from the left and right dorsal horns were pooled for neuron and blood vessel quantification. Differences were accepted to be statistically significant at p < 0.05. All errors are given as standard deviations.
Results

An in vitro assay was performed to better understand how FGF2 was released from HAMC/PLGA. Nearly 60% of all FGF2 contained in the HAMC/PLGA/FGF2 composite was released within 24 h (fig. 1). This early release included the FGF2 which was initially dispersed within HAMC as well as any burst release from the PLGA particles. After this initial burst, FGF2 was released slowly from the HAMC/PLGA/FGF2 composite for 8 days. To improve angiogenesis after SCI, pharmacologic approaches have recommended targeting the first 5–7 days post-injury, during which endogenous angiogenic processes occur [Loy et al., 2002].

After the 26 g clip injury, a cystic cavity formed in the spinal cord over the course of several days. Minimizing edema and cell death post-injury could result in reduced cavitation. HE/LFB was used to investigate overall tissue

Fig. 2. a Representative sagittal sections showing the injury cavity stained with HE and LFB at 28 days for aCSF, HAMC, HAMC/PLGA and HAMC/PLGA/FGF2 (from left to right, Scale bar = 500 μm). b Cavity volume was calculated from a series of cavity area sections for each group (n = 4 animals, mean ± standard deviation shown).

Fig. 1. Cumulative release of FGF2 from HAMC/PLGA/FGF2 over 18 days. Initial release results from FGF2 dispersed within HAMC, and continued release occurs from PLGA particles for up to 8 days.
morphology (fig. 2a) and to determine cavity volume 28 days post-injury. Mean cavity volume was similar for all groups: aCSF and HAMC controls had almost the same size cavities at $3.96 \pm 1.09$ and $3.89 \pm 0.87$ mm$^3$, respectively. Animals that received composite HAMC/PLGA had a mean cavity volume of $4.57 \pm 2.10$ mm$^3$, whereas those that received HAMC/PLGA/FGF2 showed the lowest mean cavity volume of $3.34 \pm 0.75$ mm$^3$ (fig. 2b). Notwithstanding the trend of smallest cavity volume observed when FGF2 is released locally to the injured spinal cord tissue for a prolonged time, there was no significant difference between any of the groups ($p > 0.05$). Moreover, general tissue morphology was similar in animals from all groups, where no proliferative growths were observed as a result of FGF2 delivery. We observed no difference in GFAP staining among the groups, suggesting that the astrocytic glial response was unaffected by FGF2 delivery (data not shown).

We have previously demonstrated that FGF2 delivered locally with HAMC alone penetrates the spinal cord in adequate concentrations to stimulate angiogenesis [Kang et al., 2010b]. Thus, blood vessels were counted to determine if localized and sustained delivery with HAMC/PLGA/FGF2 would lead to increased vascular density within injured spinal cord tissue 4 weeks post-injury. Counts were done in the dorsal horns, which are immediately adjacent to the injected gel. Figure 3a illustrates that statistically higher numbers of blood vessels were observed $\pm 300$ μm from the epicenter for animals that received HAMC/PLGA/FGF2. Increased vessel density was also observed for up to $\pm 900$ μm from the injury epicenter, although this increase was not statistically significant at $p < 0.05$. Representative images of blood vessels in the tissue are shown for the delivery of aCSF, HAMC, HAMC/PLGA and HAMC/PLGA/FGF2 in the dorsal horns in figure 3b, c, d and e, respectively.

**Fig. 3.** Blood vessel density in the dorsal horns increases rostrally and caudally to the injury epicenter for all groups. A statistically higher blood vessel density relative to all other controls was observed $\pm 300$ μm from the epicenter for animals that received composite HAMC/PLGA/FGF2 (n = 4 animals, mean $\pm$ standard deviation shown). Circle = aCSF; Square = HAMC; triangle = HAMC/PLGA; diamond = HAMC/PLGA/FGF2. * $p < 0.05$. Representative images of blood vessels from aCSF (b), HAMC (c), HAMC/PLGA (d) and HAMC/PLGA/FGF2 (e) in the dorsal horns. Scale bars = 100 μm.
To determine if the increased blood vessel density resulted in better neuronal survival, neurons were also counted in the dorsal horns. As observed in figure 2b, the cavity is largest at the injury epicenter where the least amount of tissue is spared from degeneration. Similar to the blood vessel counts, the lowest number of neurons was observed in the injury epicenter for all groups, reflecting the large cavity at this site. There were more surviving neurons rostral and caudal to the epicenter in all groups, but those treated with FGF2 did not have significantly greater neuron density (fig. 4a). Representative images of neurons from aCSF (b), HAMC (c), HAMC/PLGA (d) and HAMC/PLGA/FGF2 (e) in the dorsal horns. Scale bars = 100 μm.

Discussion

In SCI, immediate disruption of the neural and vascular structures occurs as a result of the primary trauma. Damaged blood vessels leak blood and fluid into tissue, and then cells and proteins, not normally present in the central nervous system, invade this damaged tissue. The local hemorrhage and edema occurring from damaged blood vessels leads to vasoconstriction around the injury [Koyanagi et al., 1993a, b], causing ischemia in the surrounding tissue penumbra and ultimately leading to extensive cell death and significant loss of function. The inflammatory process and factors from outside the central nervous system indicate a break down in the blood
spinal cord barrier, contributing further to the tissue penumbra of necrosis and apoptosis in a much larger volume than the primary trauma. Systemic FGF2 infusion causes vasodilation in vivo, by opening ATP-sensitive potassium channels and enhancing the release of nitric oxide [Cuevas et al., 1991]. However, systemic vasodilation reduces blood pressure, which is not ideal after SCI. By delivering FGF2 locally with HAMC/PLGA/FGF2 we aimed to mitigate the immediate ischemic injury without affecting systemic blood pressure. In addition, early delivery of FGF2 may also reduce the permeability of the blood-spinal cord barrier after SCI [Kang et al., 2010a], thus limiting the transfer of fluid and blood proteins into the tissue parenchyma. To target these early post-traumatic events, FGF2 was dispersed in HAMC itself to achieve quick release into the tissue, thereby limiting early vasospasm and permeability, and ultimately restricting tissue degeneration. Thus, early FGF2 release may have provided vasoprotection, leading to the increased vascular density observed near the epicenter. In addition, we observed a small reduction in cavity volume for the composite HAMC/PLGA/FGF2 group at 28 days post-injury. Though not statistically significant, it is possible that this small reduction in cavitation was due to the early FGF2 release from HAMC, limiting the ischemic injury from vasoconstriction.

Previous studies done in rats and mice have shown that endogenous adaptive angiogenesis occurs within the first week post-injury [Loy et al., 2002], and thus therapies designed to improve angiogenesis and blood flow are best suited for this time frame. Release of FGF2 from our original HAMC formulation continued for only several hours [Kang et al., 2010b], therefore a new formulation was developed utilizing PLGA nanoparticles to achieve a sustained release profile [Baumann et al., 2009]. This nanoparticle composite with HAMC was first tested for safety to ensure there were no detrimental effects of the material in the intrathecal space [Baumann et al., 2010]. Here, we have shown that FGF2 is released from the HAMC/PLGA/FGF2 composite over 8 days, encompassing the time frame of post-traumatic angiogenesis. Thus, increased vascular density may also be due to this long-term release achieved with our HAMC/PLGA/FGF2 composite in addition to the vasoprotection from early release.

The nanoparticles in the composite HAMC/PLGA/FGF2 act both as a stabilizer for the gel [Baumann et al., 2009] and as a drug depot from which FGF2 can be slowly released. These FGF2-loaded nanoparticles were incorporated into HAMC as a means to hasten and increase the angiogenic response that occurs within 7 days after SCI [Loy et al., 2002], and to improve local blood flow in the biochemically hostile environment at the injury site. Here, we have demonstrated that local and sustained delivery of FGF2 can induce angiogenesis following SCI. At 28 days post-injury, well after the peak of endogenous angiogenesis, greater blood vessel density was observed in the area ±300 μm rostral/caudal to the injury epicenter.
for animals that received FGF2 with the HAMC/PLGA/FGF2 composite. Arguably, this area nearest the epicenter of injury is most important to limit the spread of tissue degeneration that is characteristic of the secondary injury. The trend in higher density continued up to ± 900 μm away from the epicenter, consistent with our observation during surgery that HAMC can extend this length of the spinal cord when injected into the intrathecal space. Whether by angiogenesis or vasoprotection, localized sustained delivery of FGF2 from HAMC/PLGA/FGF2 results in moderately improved tissue outcome after SCI. Moreover, this strategy provides a new approach for local sustained delivery of multiple factors.

We did not observe the functional improvement previously reported when FGF2 was delivered with a minipump for 7 days post-injury [Rabchevsky et al., 1999, 2000]. However, we also did not observe the proliferative spinal cord lesions that have been reported for high doses of FGF2 delivered with minipumps [Parr and Tator, 2007]. These proliferative growths led to significant compression of the spinal cord, which could have a negative long-term impact on neurophysiology. We did not observe any proliferative growths in any of the spinal cords, particularly at the site of injection where HAMC/PLGA/FGF2 was in contact with spinal cord tissue. This is likely due to the lower dose of FGF2 delivered with composite HAMC/PLGA/FGF2 versus that delivered with minipumps. Since no dose response experiments have been performed with FGF2 to investigate the dosage and delivery rate necessary to produce angiogenesis in the spinal cord, we delivered a lower dose of FGF2 than previous minipump delivery studies to prevent excessive proliferative growths. Importantly, the initial amount of FGF2 delivered from HAMC/PLGA/FGF2 has previously been shown to penetrate into the spinal cord [Kang et al., 2010b] in concentrations comparable to other studies that have demonstrated angiogenesis with FGF2 [Montesano et al., 1986]. Improvement might be attained by either locally delivering a greater dose of FGF2 or in combination with other therapeutic molecules rather than a single factor alone. The composite HAMC/PLGA system permits the release of multiple factors and at varying rates, providing significant opportunity for the release of a cocktail of hydrophilic and hydrophobic factors of varying molecular weights [Baumann et al., 2009; Wang et al., 2009], thus providing a more diverse and safe delivery tool than the minipump/catheter.

Here, for the first time, we have shown a beneficial tissue response of local and sustained delivery of FGF2 with a biocompatible nanoparticle hydrogel composite after SCI without causing proliferative growths that have been observed with minipump/catheter delivery. Intrathecal delivery of FGF2 with the HAMC/PLGA/FGF2 composite led to significantly greater blood vessels near the injury epicenter where tissue damage is greatest and blood flow is most important to limit degenerative impacts of injury. To achieve better neuroprotection and a functional benefit, we are investigating combination strategies whereby several molecules are co-delivered with the composite HAMC.

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Disclosure Statement

No competing financial interests exist for any of the authors; however, we acknowledge that a patent has been filed for sustained release from composite HAMC.

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