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Injectable intrathecal delivery system for localized administration of EGF and FGF-2 to the injured rat spinal cord

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Abstract

The administration of growth factors (GFs) for treatment of experimental spinal cord injury (SCI) has shown limited benefits. One reason may be the mode of delivery to the injury site. We have developed a minimally invasive and safe drug delivery system (DDS) consisting of a highly concentrated collagen solution that can be injected intrathecally at the site of injury providing localized delivery of GFs. Using the injectable DDS, epidermal growth factor (EGF) and basic fibroblast growth factor (FGF-2) were co-delivered in the subarachnoid space of Sprague–Dawley rats. The in vivo distribution of EGF and FGF-2 in both injured and uninjured animals was monitored by immunohistochemistry. Although significant differences in the distribution of EGF and FGF-2 in the spinal cord were evident, localized delivery of the GFs resulted in significantly less cavitation at the lesion epicenter and for at least 720 μm caudal to it compared to control animals without the DDS. There was also significantly more white matter sparing at the lesion epicenter in animals receiving the GFs compared to control animals. Moreover, at 14 days post-injection, delivery of the GFs resulted in significantly greater ependymal cell proliferation in the central canal immediately rostral and caudal to the lesion edge compared to controls. These results demonstrate that the injectable DDS provides a new paradigm for localized delivery of bioactive therapeutic agents to the injured spinal cord.

Keywords: Diffusion; Distribution; Growth factor; Drug delivery; Subarachnoid

Introduction

Traumatic spinal cord injury (SCI) is a common and irreversible event that can incapacitate victims for life. Although the complexity of the pathophysiological processes involved in SCI have made its treatment a challenge, several experimental strategies aimed at providing neuroprotection (Agrawal et al., 2000; Bracken, 2001; Geisler et al., 1991; Rosenberg et al., 1999) and/or enhancing neuroregeneration (Bradbury et al., 2002; Brosamle et al., 2000; Fournier et al., 2003; Li and Strittmatter, 2003) have shown beneficial results. The common trend in many of these approaches has been to deliver therapeutic agents to promote recovery after SCI. Thus, efficient delivery of therapeutic agents is of utmost importance to maximize their beneficial effects.

Administration of drugs to the central nervous system (CNS) and in particular to the spinal cord has been achieved by several routes including systemic, intraparenchymal, intraventricular, and intrathecal (Thorne and Frey, 2001; Yaksh, 1999). Systemic delivery, by oral, intravenous or intraarterial administration, has been used extensively for the delivery of anti-inflammatory drugs, such as methylprednisolone (Bracken et al., 1992, 1997). Although this route of delivery has been effective in treating certain CNS conditions, it is not practical for continuous and sustained

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administration of drugs. Moreover, redistribution of systemically administered agents in the body can result in adverse side effects. Localized delivery of therapeutic agents can be achieved with intraparenchymal delivery but it is invasive to nervous tissue and may cause injury.

One of the most commonly used delivery routes in SCI research is intrathecal delivery using the osmotic minipump (Jakeman et al., 1998; Kojima and Tator, 2002; Namiki et al., 2000; Novikova et al., 2000). The minipump provides a reliable dosage of the infusate into the intrathecal space, but there are several limitations with this delivery system, including the invasiveness of implantation and induction of a chronic inflammatory response (Jones and Tuszynski, 2001; Le Breton et al., 2001; McMillan et al., 2003; Thorne and Frey, 2001; Yaksh, 1999). We have developed a novel, injectable drug delivery system (DDS) that is minimally invasive and safe (Jimenez Hamann et al., 2003). One of the advantages of using the injectable DDS is the provision for localized delivery of therapeutic agents, such as growth factors (GFs), neuroprotective agents, or gene therapies, to the injury site.

In the present study, we investigated the effects of delivering epidermal growth factor (EGF) and basic fibroblast growth factor (FGF-2) from the DDS in SCI rats. EGF and FGF-2 were selected because their co-delivery has been shown to stimulate the proliferation and differentiation of ependymal cells, a potential source of endogenous progenitor/stem cells in the adult spinal cord (Kojima and Tator, 2002; Martens et al., 2002). To determine whether the DDS provided localized delivery of the GFs, the distribution of EGF and FGF-2 in the spinal cord was assessed. The therapeutic value of delivering GFs with the injectable DDS was evaluated by histological analysis of the tissue, including quantification of cavitation and ependymal cell proliferation.

Materials and methods

In vitro release of EGF and FGF-2 from collagen gels

The delivery matrices consist of highly concentrated collagen solutions (24–28 mg/ml, Xium L.L.C, Westerly, RI) prepared from pepsin-solubilized bovine dermal collagen. These solutions gel quickly (within 1 min) when ejected into physiological, aqueous environments (pH 7.4, 37°C). Recombinant human EGF (rhEGF) alone or EGF co-dissolved with recombinant human FGF-2 (rhFGF-2) (PeproTech, Ottawa, ON) were dispersed within the collagen solution in a 1:10 ratio (v/v) yielding a final encapsulated concentration of 22.7 μg/ml for each GF. Heparin (187 USP U/mg, Sigma, Oakville, ON) was used in a 1:2 (w/w) ratio to FGF-2 to stabilize and potentiate its mitogenic activity (Edelman et al., 1991; Gospodarowicz and Cheng, 1986; Prestrelski et al., 1992). The collagen solutions containing the GFs as well those without any GFs were placed in artificial cerebrospinal fluid (aCSF, 148 mM NaCl, 3 mM KCl, 0.8 mM $MgCl_2$, 1.4 mM $CaCl_2$, 1.5 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 0.1 mg/ml of bovine serum albumin). The release experiments were conducted in aCSF (pH 7.4) at 37°C to approximate the physiological intrathecal environment. The volume ratio of collagen to aCSF was 1:10 and was chosen to approximate the volume ratio that was used in vivo. All plastic ware used was pre-coated with BSA to minimize protein adhesion.

The complete volume of aCSF (1 ml) was collected at specified times (1, 4, 7, 14, 28, and 56 days) and replaced with fresh aCSF. Half of the collected supernatant was used to measure the concentration of the released EGF and FGF-2 with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, for EGF and Chemicon International, Temecula, CA, for FGF-2) using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). The remaining collected supernatant samples were stored at −20°C and used to test the bioactivity of the released GFs. After the last collection, the collagen matrices were mechanically disrupted by vortexing and sonication, and the supernatant was also assayed to determine the amount of GF remaining in the matrix.

Bioactivity assay for released EGF

3T3/NIH fibroblasts (ATCC, Manassas, VA) were used to evaluate the bioactivity of released EGF alone, EGF/FGF-2, and control samples. 3T3/NIH cells were cultured in DMEM, 10% fetal bovine serum, and 1% antibiotic-antimycotic (Gibco BRL) in a 37°C, 5% CO_2 environment. Cells were seeded in 96 well plates at 1×10^5 cells/ml in serum-free media before addition of the GF samples. The bioactivity of the samples was evaluated by measuring cell proliferation using the MTS assay kit (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI), where the absorbance, measured at 490 nm using a Versamax plate microplate reader, is directly proportional to the number of living cells in culture. The MTS/PMS complex (20 μl/well) was added to the cells 24 h after addition of the GFs in the supernatant samples from the in vitro release experiments, and cell proliferation was measured 4 h later. In controls, cells were assayed with the supernatant collected from collagen matrices without GFs.

Bioactivity assay for released FGF-2

The rat pheochromocytoma (PC12) cell line (ATCC) was used to evaluate the bioactivity of FGF-2 by quantifying neurite lengths extending from PC12 cells in response to the supernatant samples collected from the in vitro release experiments. Although the EGF receptor is expressed in PC-12 cells, EGF stimulation does not

induce neurite extension (Chandler and Herschman, 1980; Huff et al., 1981).

PC12 cells were cultured in RPMI-1640 media (Sigma, Oakville, ON) containing 10% heat inactivated horse serum, 5% fetal bovine serum, and 1% antibiotic-antimycotic (penicillin/streptomycin/amphotericin B; Gibco BRL, Burlington, ON) at 37°C in a 5% CO_2 atmosphere. Cells were plated at 8×10^3 cells/ml in collagen-coated 96 well plates and were allowed to settle for 4 h before adding the GF samples. After 4 days of incubation with the GFs, the cells were fixed with 3.7% formaldehyde for 10 min. Cells were visualized at a final magnification of 200× using a Zeiss inverted microscope (Axiovert S100, Zeiss, Toronto, ON), and images of the cells were taken with a digital camera connected to the microscope using the Spot program (Diagnostic Instruments, Sterling Heights, MI). Ten fields were taken per well, and all experiments were done in triplicate. Released GF and control samples were evaluated to determine whether released FGF-2 remained bioactive and elicited neurite extension (≥20 μm) in the cells.

Surgical procedures

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and approved protocols from the Animal Care Committee of the Research Institute of the University Health Network. A total of 115 female Sprague–Dawley rats (250–300 g, Charles River, St Constant, Quebec) were used in this study as detailed in Table 1.

Sensitivity of detection of EGF and FGF-2 in the spinal cord

The rats (n = 14) were anesthetized by inhalation of 5% halothane and a mixture of nitrous oxide and oxygen (1:2). A heating pad was used to maintain body temperature at 37°C. A laminectomy was performed at the second thoracic (T2) spinal level with the aid of an operating microscope to expose the dura mater.

The sensitivity of the immunohistochemical technique used to monitor the distribution of rhEGF and rhFGF-2 in the rat spinal cord was evaluated by performing intramedullary injections of increasing concentrations (0, 0.1, 1, 10, and 100 μg/ml) of soluble GFs. Animals received an intramedullary injection (10 μl) of a mixture of the two GFs or aCSF over a 2-min interval using a 50-μl Hamilton syringe (Cole Parmer, Anjou, Quebec). The syringe was connected to Silastic tubing (I.D. 0.3 mm, O.D. 0.64 mm, Clay Adams, Parsippany NJ), attached to a 30-G needle bent at a 45° angle. The needle was inserted into the dorsal spinal cord lateral to the posterior sulcus. After injection, the needle was left in the spinal cord for an additional 3 min. The animals were sacrificed immediately after injection of the GFs by intraperitoneal injection of sodium pentobarbital.

Table 1
Experimental design describing the number of animals used in the study groups

Study groups	Time points						
	0 h	0.5 h	6 h	1 day	7 days	14 days	56 days
Sensitivity of detection of EGF and FGF-2 in the spinal cord							
0 μg/ml EGF and FGF-2	2						
0.1 μg/ml EGF and FGF-2	2						
1 μg/ml EGF and FGF-2	2						
10 μg/ml EGF and FGF-2	2						
100 μg/ml EGF and FGF-2	2						
100 μg/ml EGF	2						
100 μg/ml FGF-2	2						
Distribution of EGF and FGF-2 in the spinal cord (20 μl injections)							
Uninjured animals							
Collagen alone	3	3	3	3			
Collagen with 250 μg/ml of each GF	5	5	5	5			
Bolus of 250 μg/ml of each GF		3					
Severely injured animals (35 g clip compression)							
Collagen alone	3	3	3	3			4
Collagen with 250 μg/ml of each GF	5	5	5	5			4
Bolus of 250 μg/ml of each GF		3					
aCSF							3
Therapeutic effect of EGF and FGF-2 in the moderately injured spinal cord (20 g clip compression)							
Collagen with 50 μg/ml of each GF						4[a]	4
Injured, but no injection						4[a]	4
BrdU control animals (no surgery)						4[a]	

[a] Animals in the 14-day survival time point received daily injections of BrdU.

Distribution of EGF and FGF-2

Twenty microliters of either the collagen solution (alone or containing EGF and FGF-2) or aCSF was injected intrathecally at the T2 spinal level of both uninjured and injured animals using a Visitec nucleus hydrodissector (Becton Dickinson Biosciences, Mississauga, ON), as previously described (Jimenez Hamann et al., 2003) and as detailed in Table 1. Severe compressive SCI was inflicted at the T2 level of the animals by compressing the spinal cord for 1 min using a modified aneurysm clip calibrated to 35 g (Rivlin and Tator, 1978). Localized delivery of EGF and FGF-2 with the DDS was compared to bolus intrathecal injections of soluble GFs by injecting a 20-μl bolus containing EGF and FGF-2 (Table 1). The injection was performed in the same manner as the DDS injections using the nucleus hydrodissector. Bolus injections were performed in both uninjured and severely (35 g clip) injured animals.

After intrathecal injection of the DDS, the overlying muscles and fascia were sutured with 3.0 Vicryl sutures (Ethicon, Peterborough, ON), and the skin closed with

Michel clips (Fine Science Tools, Vancouver, BC). At the end of the surgery, all animals were ventilated with pure O_2 for 2–3 min and were then placed under a heating lamp during recovery from anesthesia. The rats were given buprenorphine (0.03 mg/kg) post-operatively for pain control before awakening.

Post-operative urinary tract infections in the injured animals were treated with intramuscular ampicillin (50 mg/ml) and/or gentamicin (8 mg/kg). The bladders of the SCI animals were manually evacuated three times per day until sacrifice. Animals were housed separately in a temperature-controlled room (27°C) with a 12-h light/dark cycle.

Rats were sacrificed at 30 min, 6 h, 1 day, 7 days, 14 days, or 56 days after surgery (Table 1). They were deeply anesthetized by intraperitoneal injection of sodium pentobarbital and were injected intracardially with 1 ml of 1000 U/ml heparin followed by perfusion with 10% neutral buffered formalin. The spinal cords were harvested and placed in 10% neutral buffered formalin for histological processing.

Therapeutic effect of EGF and FGF-2 administration

The therapeutic effect of EGF and FGF-2 after moderate (20 g clip) SCI was evaluated in 16 animals by following both ependymal cell proliferation using BrdU labeling and behavioral response using the Basso Beattie Bresnahan (BBB) locomotor scoring scale (Basso et al., 1996). In 8 animals, 20 μl of collagen containing 50 μg/ml of each GF were injected intrathecally immediately after injury. Control animals ($n = 8$) also underwent compression SCI but did not receive the DDS (cf. Table 1).

Animals in the 14-day survival group received daily intraperitoneal injections (50 mg/kg) of bromodeoxyuridine (BrdU) to assess the proliferation of ependymal cells after EGF and FGF-2 delivery. The BrdU injections were prepared daily with saline. An additional group of 4 animals that did not undergo surgery also received daily BrdU injections for 14 days and served as a baseline control (Table 1).

Functional evaluation was performed weekly using the BBB scoring scale. For the BBB test, each leg was observed separately, and the final score was the average of the two legs. Scores were recorded by two blinded observers and all behavioral tests were videotaped. Statistical analysis of the BBB results was performed by pooling the scores for each group at the different time points.

Histology

Formalin preserved spinal cord segments encompassing the injection and injury sites (1.5 cm) were embedded in paraffin and 8-μm-thick serial, transverse, or parasagittal sections were cut. Every tenth section was stained with non-acidic hematoxylin and eosin (H&E) for general morphology, and every eleventh section with Van Gieson's, a collagen-specific stain as previously described (Jimenez Hamann et al., 2003). Sections at the lesion epicenter were also stained with Luxol fast blue (LFB)/H&E to assess for the amount of remaining myelin after SCI. The amount of collagen deposition in the epicenter was assessed by Masson's trichrome staining. The remaining sections were used for immunohistochemistry.

EGF/FGF-2 immunohistochemical protocols

An immunohistochemical technique to track rhEGF and rhFGF-2 in paraffin embedded rat spinal cord sections was developed to assess growth factor penetration into the spinal cord. Polyclonal rabbit anti-human EGF and monoclonal mouse anti-human FGF-2 were purchased from Oncogene Research Products (San Diego, CA). Tissue sections were deparaffinized and treated with 1.2% (v/v) hydrogen peroxide in PBS for 30 min. Proteolytic digestion was performed for 20 min in an aqueous mixture of pepsin (5 mg/ml) and 2 N HCl (1% v/v) at 37°C. The sections were blocked with 1% (v/v) normal goat serum, 0.5% (w/v) BSA, and 0.3% Triton-X in PBS for 20 min. The slides were then incubated with either anti-EGF (1:100) or anti-FGF-2 (1:100) for 2 h at room temperature. The LSAB visualization kit (DakoCytomation, Mississauga, ON) was used with either 3,3′-diaminobenzidine (DAB) or VIP (Vector Laboratories, Burlington, ON) as the chromogens. This kit can be used with both rabbit and mouse primary antibodies. Control sections included spinal cord samples from animals that received intrathecal injections of collagen alone (i.e., without GFs) as well as samples in which the antibody was omitted.

Distribution of EGF and FGF-2 in the spinal cord

The extent of EGF and FGF-2 penetration into the spinal cord was quantified in two ways (Fig. 1). Images of the spinal cord were captured with the Bioquant image analysis program (R&M Biometrics, Nashville, TN) interfaced with a Nikon CCD camera mounted on a Nikon Eclipse TE 300 microscope (Tokyo, Japan). Firstly, the section with the greatest depth of penetration of the GFs into the dorsal aspect of the spinal cord was determined, and the depth of penetration was measured along a line drawn perpendicular from the dorsal edge of the spinal cord to the ventral-most edge of the tissue positive for the GF (Fig. 1). The depth of penetration into the ventral region of the spinal cord was not measured because the penetration of the GFs was not as extensive as in the dorsal region. Measurements were normalized by dividing the recorded depth by the width of the spinal cord at the same location, and the results recorded as percent penetration. The second method of quantification of GF penetration into the spinal cord was based on the area of tissue positive for the GF and was obtained by manually tracing these areas in both the dorsal and ventral portions of the spinal cord (Fig. 1). The areas positive for GF

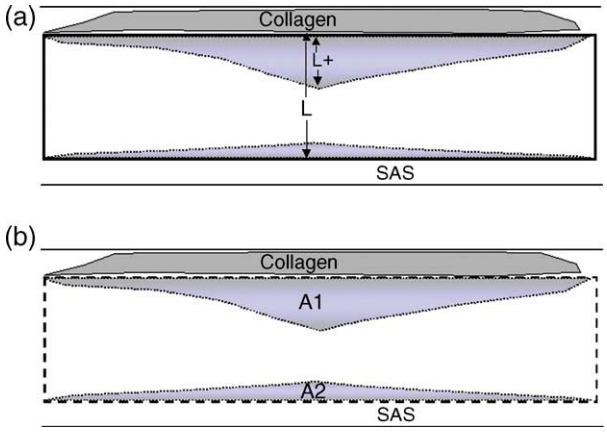

Fig. 1. (a) Schematic of a parasagittal spinal cord section. The colored areas denote where the spinal cord is positively stained for rhEGF as determined by immunohistochemistry. The location of greatest depth of penetration of EGF is denoted as L+, and the maximum diameter of the spinal cord at that site is denoted as L. The maximal percent depth of penetration was determined by dividing L+ by L and multiplying by 100. (b) For determination of the maximal area of distribution, the colored areas positive for rhEGF, A1 and A2, were added, and the sum of A1 and A2 was then divided by the total area of the spinal cord section in which rhEGF was detected as shown by the dashed line.

penetration were added together and divided by the total cross-sectional area of the spinal cord at the same site and expressed as percent of maximum area of penetration of the GFs.

Analysis of the epicenter

Cavitation was quantified in animals receiving the GFs and in control animals at 56 days after SCI. Sections stained with H&E were imaged using a 2× objective (final magnification 20×) that allowed visualization of the cross-section of the entire spinal cord. Cavitation was quantified by measuring the area of the cavity using the manual fill tool in Sigma Scan (v.1.20) and dividing this value by the total cross-sectional area of the spinal cord, including the cavity. Measurements were made every 80 μm for a distance of 2160 μm rostral and caudal to the epicenter. Due to the large number of sections, data were binned for 6 successive sections comprising a distance of 480 μm. The section with the maximum amount of cavitation or with the least amount of tissue preservation was considered as the lesion epicenter.

At the lesion epicenter, the amount of white matter spared was analyzed in sections stained with LFB/H&E. Sections positive for blue stained myelin compared to non-myelinated sections that stained pink. The white matter areas were outlined using the manual fill tool in Sigma Scan and divided by the total cross-sectional area of the spinal cord at that site. The astrocytic and inflammatory responses were also analyzed at the epicenter with glial fibrillary acidic protein (GFAP) and ED-1 immunohistochemistry, respectively. Both monoclonal mouse anti-GFAP and monoclonal mouse anti-ED-1 were purchased from Chemicon International, Inc. (Temecula, CA). The antibodies were used as previously described (Jimenez Hamann et al., 2003; Kojima and Tator, 2002) with VIP (Vector Laboratories, Burlington, ON) as the chromogen. Since astrocytes, macrophages, and microglia often form cell clusters after SCI, it is difficult to count individual cells labeled with GFAP or ED-1 (Popovich et al., 1997). The areas positive for GFAP at the epicenter were outlined with the manual fill tool in Sigma Scan and divided by the total cross-sectional area at that location. The inflammatory response to localized delivery of EGF and FGF-2 was assessed by measuring the total areas positive for ED-1 immunoreactivity present in the dorsal funiculus at the lesion epicenter. The results were normalized by dividing them by the total tissue area at the same site.

Ependymal cell proliferation

The proliferative activity of ependymal cells after EGF and FGF-2 delivery was assessed with biotinylated horse anti-mouse BrdU (Becton Dickinson, Mississauga, ON). The antibody was used as previously described (Kojima and Tator, 2002) with DAB as the chromogen for BrdU reacted sections, and Mayer's hematoxylin as the counterstain.

Tissue sections were analyzed using light microscopy, and the central canal was visualized at a final magnification of 400× in order to accurately count the cells. The labeling index (LI) was obtained by dividing the BrdU-labeled ependymal cells by the total number of cells situated around the central canal. Measurements were made in the section closest to the epicenter in which there was an intact central canal, and this was done rostral and caudal from the lesion epicenter. Measurements were made every 80 μm and a minimum of 112 tissue sections per animal were manually counted. Due to the large number of measurements, the results were binned for every 1120 μm segment from the epicenter.

Results

In vitro release of EGF and FGF-2 from collagen gels

Greater than 95% of the original amount of EGF and FGF-2 added to the collagen solution was incorporated into the matrix. Colorimetric ELISAs were used to monitor the release profile of EGF and FGF-2 from collagen matrices (Fig. 2). EGF was rapidly released from the collagen matrix with approximately 67% being released in 4 days. In contrast, FGF-2 release was more prolonged, with approximately 55% released in 14 days. At the end of the experiment (56 days), EGF could not be detected in the collagen matrix while ~35% of FGF-2 was still present in the matrix.

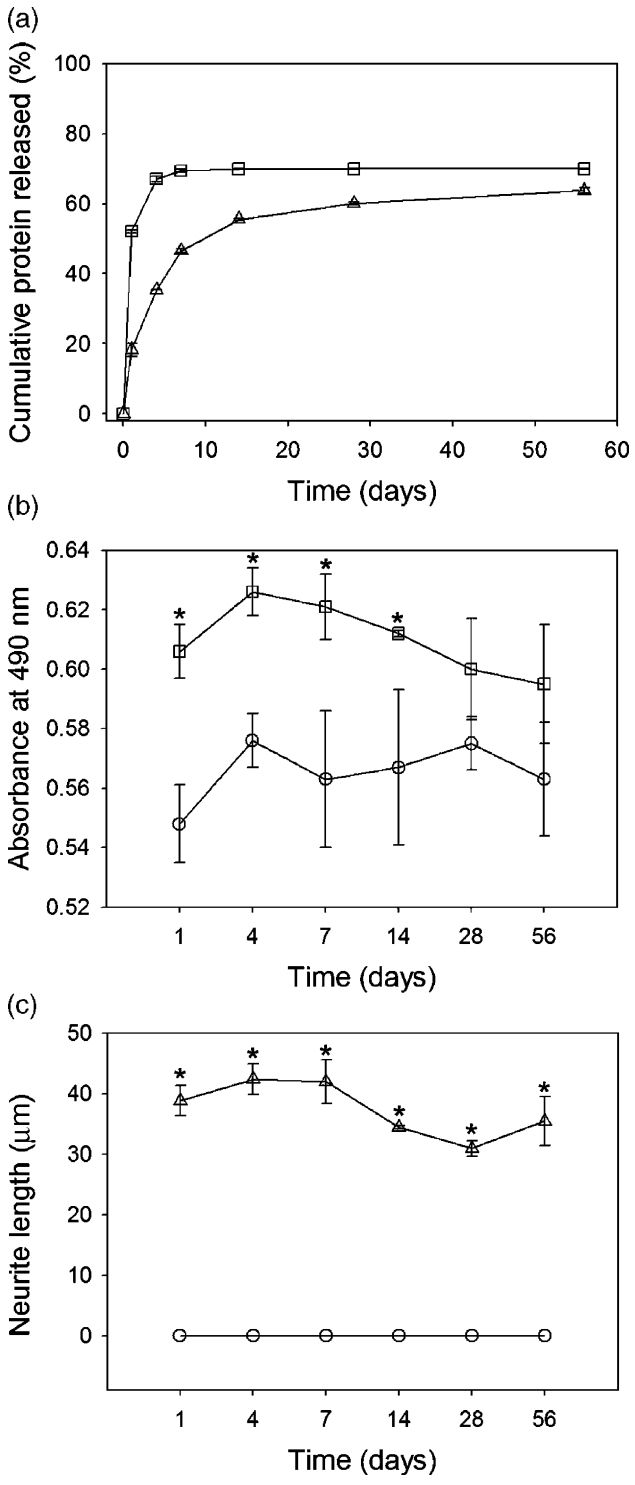

Fig. 2. (a) Cumulative release of EGF (□) and FGF-2 (Δ) from the collagen matrices monitored with ELISA. (b) The bioactivity of released EGF from the collagen matrices was assessed with 3T3/NIH fibroblasts using an MTS/PMS-based cell proliferation assay. EGF (□) released in the samples was bioactive and significantly different from controls (○) for the first 14 days of the experiment (t test, $^*P < 0.05$). (c) The bioactivity of FGF-2 released in the samples was assessed with PC-12 cells extending neurites with lengths >20 μm. All FGF-2 (Δ) released in the samples was bioactive resulting in neurite extension and was significantly different from controls (○) (t test, $^*P < 0.05$). Means ± standard deviations are reported. All samples were tested in triplicate.

Bioactivity of released EGF and FGF-2

The bioactivity of EGF released into aCSF in vitro was tested by measuring the proliferation of 3T3/NIH fibroblasts where the absorbance from the MTS assay reflected proliferation. Released EGF collected during the first 14 days of the experiment was found to induce significantly more proliferation in fibroblasts than samples collected from control, non-EGF containing matrices (t test, $P < 0.05$) (Fig. 2). These results indicate that the released EGF remained bioactive (for at least 14 days) and was not affected by its encapsulation into the injectable DDS.

The bioactivity of FGF-2 released from the collagen matrices was tested using a PC-12 cell neurite extension assay. FGF-2 released at all time points over 56 days was bioactive and elicited neurite extension (>20 μm) in the cells compared with non-FGF-2 containing control matrices (Fig. 2). The retained FGF-2 that was not released also remained bioactive after 56 days indicating that the collagen matrix provided a stable environment for FGF-2.

Sensitivity of the EGF and FGF-2 immunohistochemical protocol

Our immunohistochemical protocol for the detection of rhEGF and rhFGF-2 in the rat spinal cord showed that neither anti-rhEGF nor anti-rhFGF-2 cross reacted with endogenous rat EGF and FGF-2 (Fig. 3). Moreover, in spinal cord samples injected with aCSF, there was no reaction when tested with anti-rhEGF and anti-rhFGF-2. There was also no cross-reaction when anti-rhEGF was tested in animals receiving rhFGF-2 alone and similar results were seen when anti-rhFGF-2 was used in spinal cord sections from animals receiving rhEGF alone. These results indicate the specificity of our immunohistochemical protocol.

After direct intramedullary injection, EGF was detected in the spinal cord at concentrations of 1–10 μg/ml and for FGF-2 at 10 μg/ml. Since 10 μl was injected into the spinal cord, the minimum amount of EGF detectable corresponds to 10–100 ng whereas for FGF-2 the minimum amount corresponds to 100 ng. At lower concentrations, neither EGF nor FGF-2 could be visualized. Although EGF and FGF-2 were injected simultaneously, their distribution in the spinal cord was significantly different (Fig. 3). EGF spread radially outward from the injection site and could be found in the white and gray matter whereas FGF-2 could only be detected immediately adjacent to the injection site. In all samples examined, FGF-2 did not diffuse as extensively as EGF.

Distribution of EGF and FGF-2 in the spinal cord delivered by the DDS

The penetration of rhEGF and rhFGF-2 delivered from the injectable DDS was examined with immunohistochemistry in formalin fixed spinal cord sections. Penetration of EGF into the spinal cord was assessed by measuring the

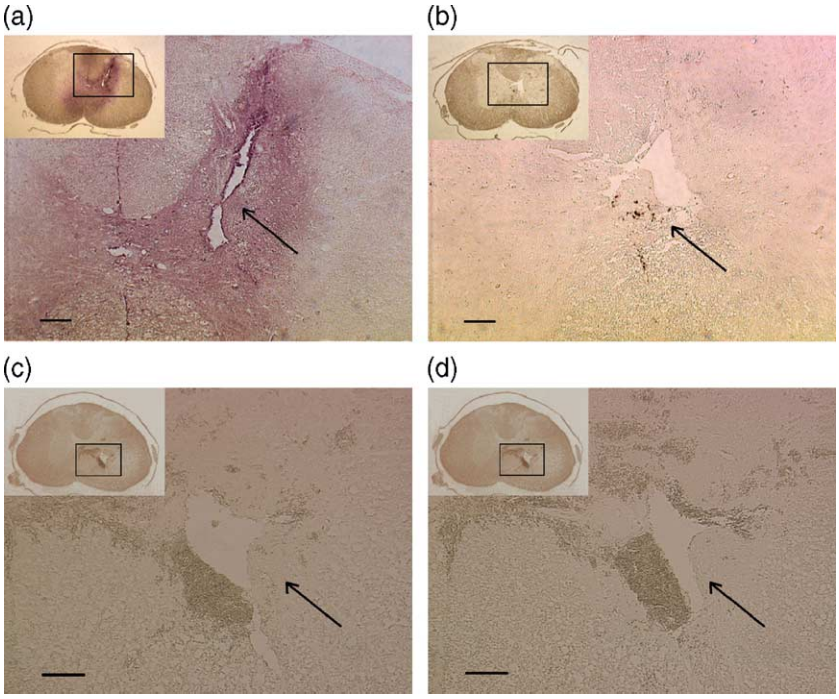

Fig. 3. (a) Intramedullary injection of rhEGF in the rat spinal cord is detectable at a minimum concentration of 10 μg/ml using VIP. (b) Detection of rhFGF-2 is also possible at 10 μg/ml using DAB. (c) Section of a spinal cord injected with aCSF and reacted with anti-rhEGF demonstrating that there is no cross-reaction with endogenous EGF. (d) Similar results were found when a spinal cord injected with aCSF was reacted with anti-rhFGF-2. The arrows indicate the needle track at the site of injection. The inset figures show cross-sectional views of the spinal cords and the rectangle in the inset shows the locations where the images were magnified. Scale bars = 100 μm.

maximal depth and area of penetration. At 30 min, EGF could be detected in both the collagen matrix and the spinal cord of SCI and uninjured rats (Fig. 4). EGF penetrated significantly deeper into the injured spinal cord at 30 min (65.6 ± 9.7%) than into the uninjured spinal cord (34.3 ± 3.0%) as determined by a *t* test (95% confidence interval, $P = 0.006$). In uninjured animals, EGF was mainly distributed in the rostral–caudal plane along the spinal cord. However, there was no significant difference in the maximum area of distribution of EGF in injured (20.7 ± 7.8%) and uninjured (27.4 ± 0.9%) animals at 30 min ($P = 0.216$, *t* test).

At 6 h after DDS injection, both the depth (62.4 ± 5.9%) and area (21.3 ± 16.5%) of EGF penetration in the injured spinal cords were similar to that observed at 30 min. In the injured spinal cords, there was no significant difference between 30 min and 6 h in terms of the maximal depth ($P = 0.655$, *t* test) and area ($P = 0.957$, *t* test) of EGF penetration. In contrast, in the uninjured animals, the maximal depth (3.5 ± 4.0%) and area (0.2 ± 0.4%) of penetration of EGF into the spinal cord decreased significantly between 30 min and 6 h (Fig. 4). In uninjured animals, the maximal depth of EGF penetration decreased approximately 10 times while the maximal area of penetration decreased about 100-fold between 30 min and 6 h. At 24 h, only a small amount of EGF could be detected in the collagen and dorsal region of the spinal cord of injured and uninjured animals. Seven days post-injection, EGF could not be detected in either the collagen matrix or the spinal cord.

The distribution of FGF-2 in the spinal cord was significantly different from that of EGF. At all time points analyzed from 30 min to 7 days, FGF-2 could only be detected in the collagen matrix and the adjacent pia and dura mater but not in the spinal cord beneath the pia (Fig. 5) of either SCI or uninjured rats. FGF-2 was retained in the collagen matrix longer than EGF, and there was a significant amount still present at 1 day. However, at 7 days, only small localized points within the collagen matrix were positive for FGF-2.

Intrathecal bolus delivery of GFs

Injured and uninjured animals received intrathecal bolus solutions of EGF and FGF-2 at the same concentrations as those delivered from the DDS. In contrast to the GFs in the DDS, neither EGF nor FGF-2 could be found in the spinal cord, pia, or dura of the analyzed tissues 6 h post-injection in either SCI or uninjured animals (Fig. 5). Injection of soluble factors (without the DDS) directly into the intrathecal space is not a reliable delivery method since the injectate may leak from the puncture site decreasing the total volume injected. While care was taken to inject the soluble GFs slowly and the needle was maintained in the intrathecal space for an additional 2 min after injection, backflow from the puncture site could not be completely

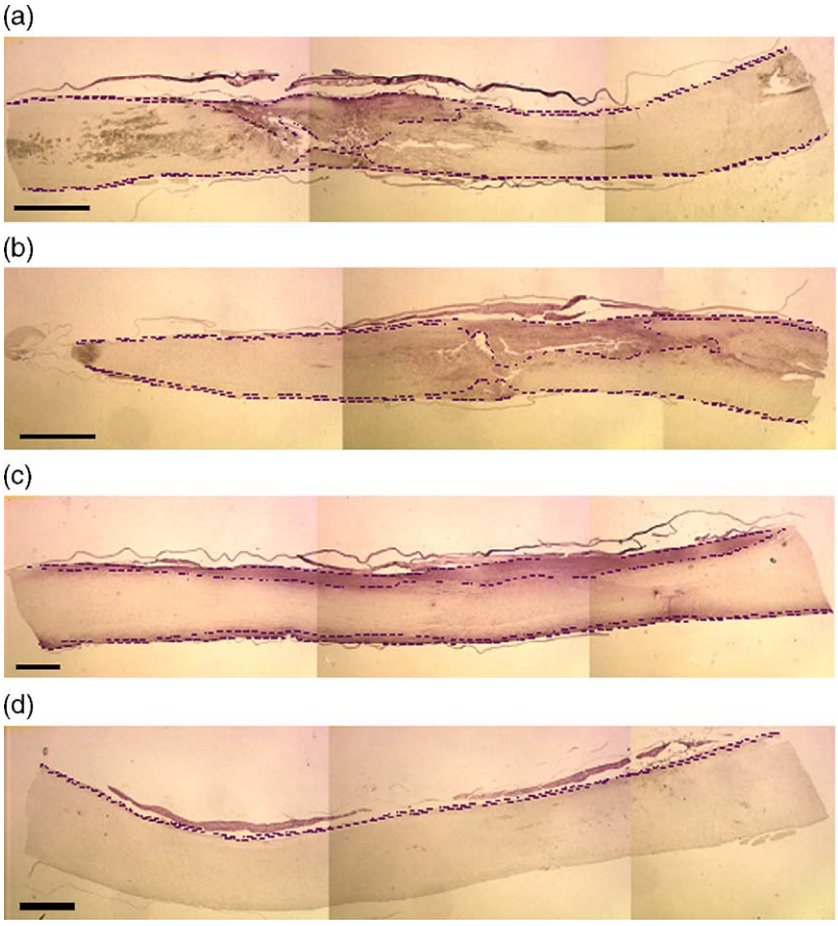

Fig. 4. In SCI animals, both at (a) 30 min and (b) 6 h, rhEGF was present to a considerate depth and was primarily localized at the injury site. (c) In contrast, rhEGF immunoreactivity 30 min post-injection in an uninjured animal showed much less penetration. (d) At 6 h, there was significantly less rhEGF in the dorsal spinal cord of an uninjured animal than at 30 min, and there was no detectable rhEGF ventrally. The chromogen used was VIP. The dashed lines represent areas in the spinal cord positive for rhEGF. Scale bars = 1 mm.

avoided. Thus, greater GF penetration into the spinal cord can be achieved with the DDS since the GFs are confined within the matrix preventing their leakage into the epidural space.

Analysis of the epicenter

The collagen matrix persisted in the subarachnoid space for at least 56 days and was found over the dorsal aspect of the spinal cord, partially filling the subarachnoid space. It spread a total distance ranging from 7.5 to 12.6 mm rostro-caudally along the dorsal aspect of the cord. Cellular invasion into the DDS containing EGF and FGF-2 was evident at 7 days with the majority of the invading cells appearing to be fibroblasts, and with a small number of macrophages and endothelial cells. When collagen alone without GFs was injected, a cellular capsule formed around the collagen but there was minimal cellular invasion into the matrix. The amount of cellular infiltration into the DDS in animals receiving collagen alone or with GFs did not increase between 14 and 56 days post-surgery.

Cavitation was compared in H&E stained spinal cord cross sections of moderately injured animals (20 g clip) that received the DDS containing EGF and FGF-2 and control animals that did not receive any injections. At the lesion epicenter spanning 480 μm longitudinally, there was significantly less cavitation in animals receiving EGF and FGF-2 compared to the control group as evaluated with a Mann–Whitney rank test used for non-normally distributed data ($P = 0.008$) (Fig. 6). There was also significantly less cavitation ($P = 0.006$) in the GF group compared to the controls caudal to the lesion epicenter. These results indicate that EGF and FGF-2 promoted tissue sparing or enhanced repair at the lesion epicenter and for at least 720 μm caudal to it.

The lesion epicenter, spanning 480 μm rostro-caudally, was also analyzed to determine the extent of white matter sparing after injury. At 56 days post-injury, animals receiving the DDS containing EGF and FGF-2 had significantly more (t test, $P < 0.05$) residual white matter (19.5 ± 2.2%, mean ± standard error) compared to control animals (12.9 ± 1.8%) (Fig. 6). Thus, localized delivery of EGF and FGF-2 promoted white matter sparing at the lesion

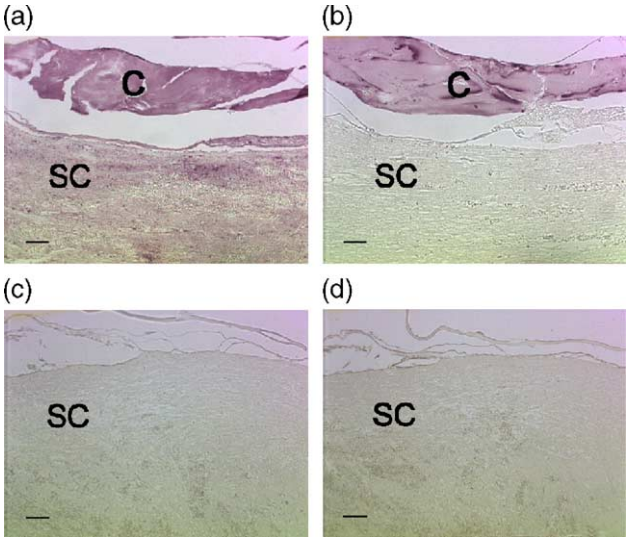

Fig. 5. At 6 h post-injection of the DDS, (a) rhEGF and (b) rhFGF-2 were detected in the collagen matrix (C) of SCI animals. Whereas rhEGF immunoreactivity was detected in the spinal cord (SC) of injured animals in panel a, no rhFGF-2 is detected in the adjacent spinal cord in panel b. The separation of the collagen matrix from the spinal cord is an artifact that occurred during processing. In contrast, 6 h after bolus intrathecal injections of the GFs, neither (c) rhEGF nor (d) rhFGF-2 can be detected in the injured spinal cord, pia, or dura. The chromogen used was VIP. Scale bars = 100 μm.

epicenter. Interestingly, the astrocytic response at this location was significantly greater in animals after EGF/FGF-2 delivery (29.8 ± 2.7%, mean ± standard error) than in control animals (22.3 ± 2.0%, *t* test, $P < 0.05$) (Fig. 6).

Further analysis of the epicenter with Masson's trichrome revealed that delivery of EGF and FGF-2 did not result in enhanced collagenous scar formation. The inflammatory response at this location was also assessed with ED-1 immunoreactivity and was found to be similar for animals receiving the GFs (4.8 ± 3.4%, mean ± standard deviation) and control animals (5.3 ± 2.9%, *t* test, $P = 0.293$) indicating that delivery of the GFs with the DDS did not induce a significant inflammatory response in the animals.

Ependymal cell proliferation

BrdU labeling indices (LIs) for ependymal cells around the central canal were quantified from the site of the first intact central canal (0 μm) to 4480 μm rostrally and caudally from the lesion edge with the results binned every 1120 μm. At 14 days, the LIs in the GF and control groups from 0 to 1120 μm rostral to the lesion epicenter were 27.7 ± 2.5% (mean ± standard error) and 11.3 ± 1.3%, respectively (Fig. 7) and significantly different (Mann–Whitney rank sum test, $P < 0.001$). Immediately caudal to the lesion

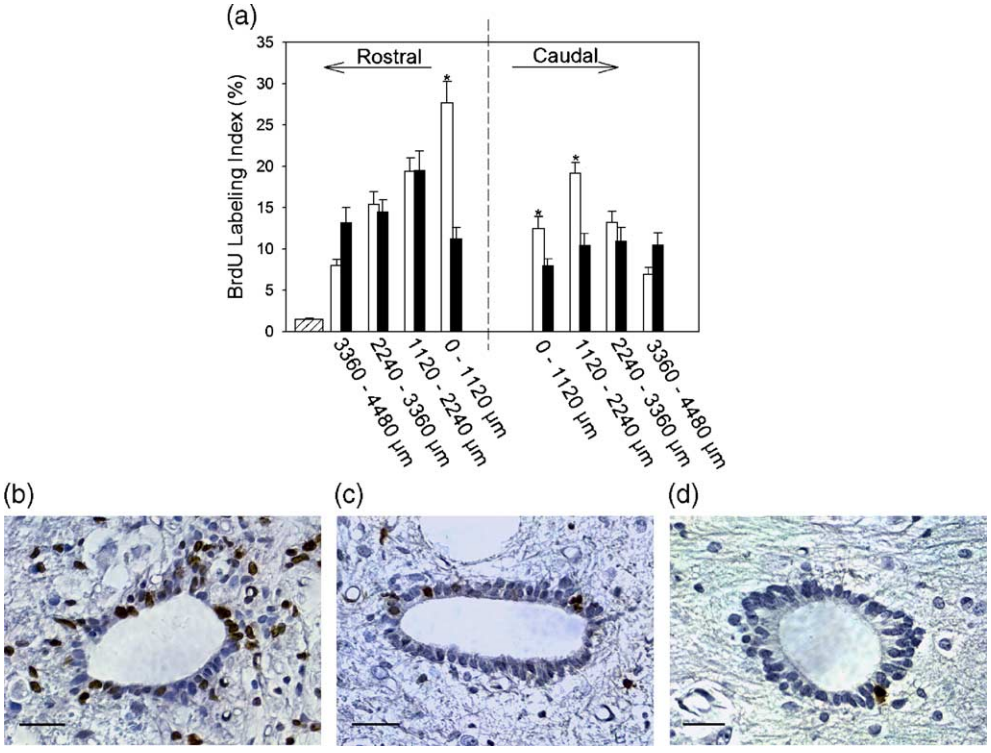

Fig. 7. (a) BrdU labeling index (LI) 14 days after compressive SCI. Animals that received the GFs ($n = 4$) are represented with white bars and control animals with black bars ($n = 4$). The hatched bar represents the LI for the intact rat spinal cord determined at the T2 spinal level ($n = 4$). Measurements of LI were made beginning at the site of the first section containing an intact central canal rostral and caudal to the lesion epicenter (dashed line). Means ± standard errors are reported. Asterisks represent significant differences, $P < 0.05$. Immunohistochemical staining of BrdU positive cells in the central canal of (b) injured animals receiving the GFs via the DDS, (c) injured control animals, and (d) in the intact spinal cord. Sections in panels b and c were 880 to 960 μm rostral to the lesion epicenter. The chromogen used was DAB and the counterstain was hematoxylin. Scale bars = 25 μm.

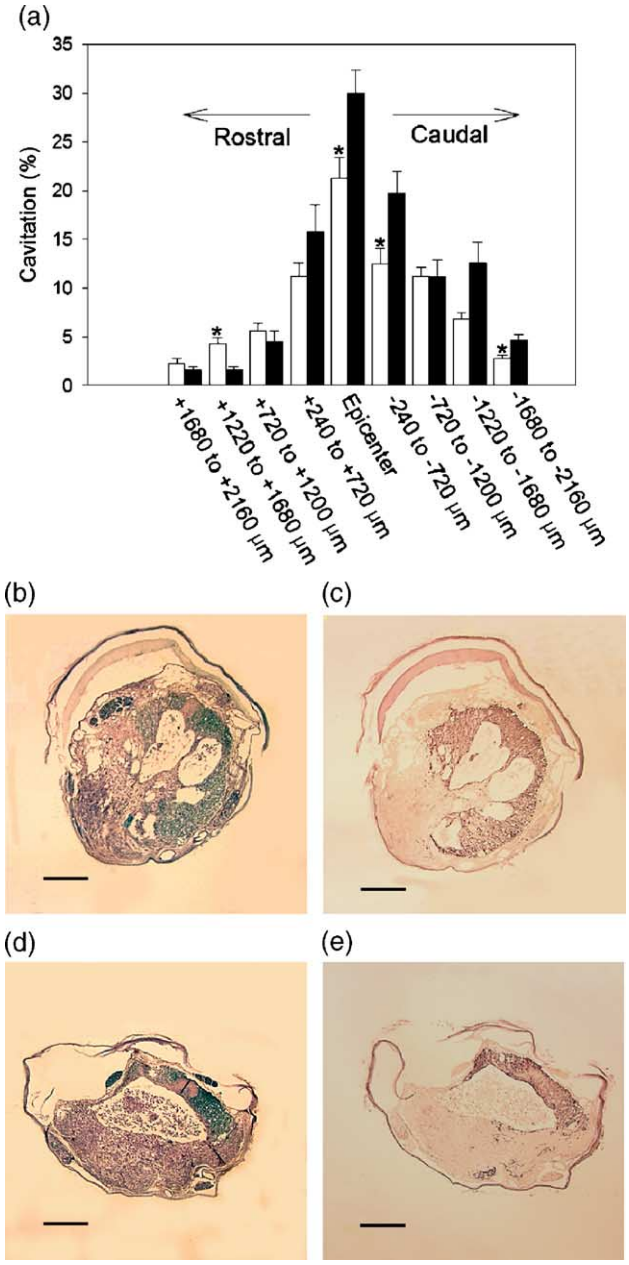

Fig. 6. (a) Percent cavitation 56 days after compressive SCI. Animals receiving EGF and FGF-2 (n = 4) are represented with white bars and controls with black bars (n = 4). The epicenter of the lesion (480 µm in length) is the site of maximum tissue loss. Means ± standard errors are reported. Asterisks represent significant differences ($P < 0.05$). Spinal cord cross sections at the injury epicenter of animals receiving the DDS containing EGF and FGF-2 (b and c) and of control animals (d and e) that did not receive any injections. The spinal cord sections were stained with either (b and d) Luxol Fast Blue or (c and e) immunohistochemically reacted for GFAP. Note that there is much more white matter sparing and greater GFAP immunoreactivity in animals that received the GFs via the DDS compared to control animals. Scale bars = 500 µm.

epicenter and continuing for up to 2240 µm, animals receiving EGF and FGF-2 also had significantly higher LIs ($P < 0.05$) than controls. The LI for the intact spinal cord (1.5 ± 0.1%) was significantly lower than that of SCI animals and was consistent with previously reported values from this laboratory and elsewhere (Horner et al., 2000; Kojima and Tator, 2002; Namiki and Tator, 1999; Takahashi et al., 2003).

Behavioral analysis

Locomotor function of moderately injured animals (20 g clip) injected with the DDS containing EGF and FGF-2 and of non-DDS controls was assessed weekly with the BBB scale. At 56 days, animals receiving GFs via the DDS (average pooled legs, n = 8) had a final BBB score of 7.9 ± 0.4 (mean ± standard deviation) and control animals had a final BBB score of 9.1 ± 1.8. There was no significant difference between groups (t test, P = 0.130).

Discussion

In this study, the efficacy and efficiency of localized delivery of EGF and FGF-2 to the injured spinal cord via a novel injectable DDS was investigated. The release of the GFs from collagen was studied first in vitro to determine their release profiles and stability after incorporation into the DDS. EGF was released rapidly from the collagen matrix while the release of FGF-2 was slower and more sustained. Ionic interactions between FGF-2 and several delivery matrices including agarose, gelatin, fibrin, and matrigel have been reported (Dabin and Courtois, 1991; Flaumenhaft et al., 1990; Tabata et al., 1994, 1998). Although interactions between FGF-2 and collagen matrices have not been formally studied (Wissink et al., 2000), non-specific ionic interactions between charged amino acid residues of collagen and FGF-2 can occur and may explain why FGF-2 was released at a slower rate compared to EGF. Both GFs remained bioactive after incorporation into the DDS indicating that the DDS provides a stable environment for EGF and FGF-2. In previous release experiments (data not shown) in which heparin was not used, FGF-2 remained bioactive for only 7–14 days. Heparin has been shown to stabilize FGF-2, protect it from enzymatic degradation, and potentiate its mitogenic effects (Edelman et al., 1991; Gallagher, 1994; Gospodarowicz and Cheng, 1986; Prestrelski et al., 1992; Safran et al., 2000; Sommer and Rifkin, 1989). Thus, heparin was considered to be an important factor in sustaining the bioactivity of FGF-2.

In vivo analysis of the distribution of EGF and FGF-2 in the spinal cord demonstrated significant differences in the penetration of the GFs. EGF readily penetrated the spinal cord shortly after injection of the DDS, whereas FGF-2 was only detected in the pia and dura. The limited diffusion of radio-labeled FGF-2 after intraventricular or intraparenchymal injections into rat nervous tissue has been previously reported (Gonzalez et al., 1994). After intraparenchymal injection, FGF-2 was detected mainly around the injection site, and after intraventricular delivery, rapid clearance of

FGF-2 from the infusion site was observed. Gonzalez et al. (1994) hypothesized that the limited diffusivity of FGF-2 may be due to its high affinity for heparan sulfate and its sequestration in the extracellular matrix.

Differences in the distribution of other GFs in the CNS have also been reported (Anderson et al., 1995; Thorne and Frey, 2001). After intracerebral bolus injections or continuous infusion into the lateral ventricle of brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3), significant differences in the penetration of these GFs into the brain were found to be unrelated to the site and method of delivery (Anderson et al., 1995). For example, NGF was readily distributed around the delivery site whereas the penetration of BDNF into brain tissue was substantially less, being confined mainly to the site of delivery. The differences in the distribution of these infused GFs into CNS tissue did not appear to be related to molecular weight or isoelectric point (Thorne and Frey, 2001), since these GFs have similar molecular weights (26,500–27,000 Da) and isoelectric points (9.5–10). Other factors limiting the diffusion of the GFs into the parenchyma appear to be involved, such as high levels of TrkB receptor binding BDNF (Anderson et al., 1995). It is important to note that while we demonstrated specificity in the immunohistochemical technique that we developed to detect EGF and FGF-2 in the paraffin-embedded spinal cord sections, this technique has limited sensitivity of detection and requires concentrations of approximately 10–100 ng of EGF and 100 ng of FGF-2 at a given time point. Increasing the sensitivity of detection with radio-labeled GFs or by alternative perfusion techniques for tissue fixation (Beckstead, 1994) may allow for more accurate determination of FGF-2 delivered to the spinal cord. In our study, we also found differences in the distribution of EGF in injured and uninjured rats. In the injured spinal cord, the distribution of EGF was mainly restricted to the injury site, where a small cavity-like region containing severely damaged tissue is formed. Thus, EGF may have persisted in the injured spinal cord due to the stagnant nature of CSF at the injury site and/or to low blood flow in the region caused by secondary ischemic damage (Tator and Fehlings, 1991). In contrast, in uninjured animals, EGF was mainly distributed along the rostro-caudal axis of the spinal cord and may then have decreased due to its rapid clearance by CSF flow. Oldfield's group also reported differences in the distribution of macromolecules between the normal and traumatized spinal cords of several species and showed that in the normal spinal cord, the infusate travels along the dorsal columns which function as low resistance pathways. (Lonser et al., 1998; Wood et al., 1999). However, after injury, the pathways in the dorsal columns are disrupted and this may allow greater penetration of the infusate into the dorsal region of the spinal cord.

Although the release of the GFs from the DDS was rapid, the delivery of EGF and FGF-2 from the DDS was more effective than bolus intrathecal injection of the GFs. After bolus injection, neither EGF nor FGF-2 could be detected in the spinal cord, pia, or dura in either SCI or uninjured animals. While the DDS serves as an intrathecal depot for bioactive GFs, the efficiency of delivery of the current DDS design is limited by its rapid release of GFs as demonstrated by our in vitro and in vivo results. The release rate can be prolonged by incorporating, into the injectable matrix, microspheres (Cao and Shoichet, 1999), and/or liposomes (Mantripragada, 2002) which have been shown to provide sustained release of GFs. Microspheres/liposomes may also allow greater loading of the GF in the matrices. Moreover, a variety of therapeutic agents, with different release rates can be included in this DDS strategy. For example, methylprednisolone, which does not need prolonged delivery, can be dispersed within the matrix for rapid release along with the GF-containing microspheres.

Analysis of the epicenter

The efficacy of localized delivery of EGF and FGF-2 to the injured spinal cord was demonstrated by a decrease in cavitation at the lesion epicenter and caudal to it. Cavitation at the site of injury is a recognized phenomenon after traumatic SCI in rats and humans (Brook et al., 1998; Fitch et al., 1999; McDonald, 1999; Schwab and Bartholdi, 1996), and we and others have shown that delivery of GFs into the injured spinal cord can decrease cavitation (Lee et al., 1999; Namiki et al., 2000; Rabchevsky et al., 1999). Lee et al. (1999) reported a reduction in the size of the lesion after intramedullary infusion of FGF-2. Although a decrease in cavitation was not accompanied by motor functional recovery, as assessed by the BBB test, this may reflect the BBB test itself more than overall functional recovery as we did not evaluate recovery in SCI animals for sensory recovery or bladder control or other motor tests (Metz et al., 2000). Thus, tissue sparing is important because it may translate into sparing of important sensory and motor axonal pathways in the spinal cord.

We found that localized delivery of EGF and FGF-2 with the DDS resulted in greater white matter sparing in the lesion epicenter compared to controls. The exact mechanism by which EGF and FGF-2 may have protected the white matter is not known but it has been shown that both astrocytes and oligodendrocytes express receptors for both EGF and FGF-2 (Bayatti and Engele, 2002; Gomez-Pinilla et al., 1988; Nieto-Sampedro et al., 1988; Plata-Salaman, 1991; Redwine et al., 1997; Reilly and Kumari, 1996; Simpson et al., 1982). The presence of the FGF-2 receptor, FGFR2, in oligodendrocytes has been hypothesized to indicate that FGF-2 is a survival factor for these cells rather than a mitogenic factor since oligodendrocytes have minimal proliferative capacity (Redwine et al., 1997). Moreover, it has also been demonstrated that FGF-2 can

prevent apoptosis in oligodendrocytes in vitro (Yasuda et al., 1995). Since FGF-2 stabilizes calcium homeostasis after hypoglycemic insult, it may also protect neurons and oligodendrocytes from increased calcium present after SCI (Cheng and Mattson, 1991; Rabchevsky et al., 1999, 2000).

The effect of localized delivery of EGF and FGF-2 on ependymal cell proliferation was also assessed. EGF and FGF-2 were selected for delivery due to their synergistic effect in promoting proliferation of ependymal cells and functional recovery in the adult rat spinal cord (Kojima and Tator, 2000, 2002). Since ependymal cells are considered to be a potential source of adult stem/progenitor cells (Johansson et al., 1999), localized delivery of EGF and FGF-2 to the injured spinal cord may improve the outcome after compressive SCI. Delivery of the GFs by the DDS promoted ependymal cell proliferation both rostral and caudal to the lesion edge. Kojima and Tator (2002) also reported increased ependymal cell proliferation in the injured spinal cord immediately caudal to the injury cavity after intrathecal infusion of EGF and FGF-2 (5 μg of each GF over 14 days) via the osmotic minipump. The present results confirm the effectiveness of localized delivery of EGF and FGF-2 with the DDS for stimulating ependymal cell proliferation in the central canal. While we were unable to detect FGF-2 in the spinal cord due to limitations in the sensitivity of the immunohistochemical protocol, some FGF-2 penetration into the spinal cord probably occurred because Kojima and Tator (2002) showed that only concomitant delivery of EGF and FGF-2 led to increased ependymal cell proliferation, a response that was not observed when only one of these GFs was delivered.

In this study, we have demonstrated the safety and potential therapeutic benefits of localized delivery of EGF and FGF-2 with the injectable DDS. The present study also provides important insights into the fate of locally delivered GFs, and their cellular effects in the injured spinal cord. The intrathecal DDS is a promising, alternative method for localized delivery of therapeutic agents to the injured spinal cord that can overcome the limitations of bolus and minipump delivery.

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