Significant reduction in neural adhesions after administration of the regenerating agent OTR4120, a synthetic glycosaminoglycan mimetic, after peripheral nerve injury in rats

Technical note

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Object. Extradural and intraneural scar formation after peripheral nerve injury frequently causes tethering and compression of the nerve as well as inhibition of axonal regeneration. Regenerating agents (RGTA) mimic stabilizing and protective properties of sulphated glycosaminoglycan toward heparin-binding growth factors. The aim of this study was to assess the effect of an RGTA known as OTR4120 on extraneural fibrosis and axonal regeneration after crush injury in a rat sciatic nerve model.

Methods. Thirty-two female Wistar rats underwent a standardized crush injury of the sciatic nerve. The animals were randomly allocated to RGTA treatment or sham treatment in a blinded design. To score neural adhesions, the force required to break the adhesions between the nerve and its surrounding tissue was measured 6 weeks after nerve crush injury. To assess axonal regeneration, magnetoneurographic measurements were performed after 5 weeks. Static footprint analysis was performed preoperatively and at Days 1, 7, 14, 17, 21, 24, 28, 35, and 42 postoperatively.

Results. The magnetoneurographic data show no significant difference in conduction capacity between the RGTA and the control group. In addition, results of the static footprint analysis demonstrate no improved or accelerated recovery pattern. However, the mean pullout force of the RGTA group (67 ± 9 g [mean ± standard error of the mean]) was significantly (p < 0.001) lower than that of the control group (207 ± 14 g [mean ± standard error of the mean]).

Conclusions. The RGTA strongly reduce nerve adherence to surrounding tissue after nerve crush injury. (DOI: 10.3171/JNS/2008/109/11/0967)

KEY WORDS • adhesion • glycosaminoglycan • heparin-binding growth factor • peripheral nerve injury • rat • regeneration
ral signals without preventing them from providing their natural stimulatory functions. They are characterized by specific activity at injured sites. This was demonstrated in a model of muscle healing after total crushing using an intravenously injected, radiolabeled RGTA. The RGTA's were readily eliminated in a noninjured rat, whereas in animals after muscle crushing, they remained solely localized in the damaged muscle until regeneration was complete. In various experimental animal models, RGTA's have been proven to reduce fibrotic tissue formation and to promote tissue remodeling and healing, for example in bone regeneration, skin repair, colonic anastomosis, and muscle regeneration. In addition, in vitro studies have demonstrated that RGTA's enhance the bioavailability of heparin-binding growth factors and regulate collagen synthesis.

We hypothesized that RGTA's may decrease extraneuronal scarring after nerve injury and enhance neural regeneration by binding natural signals at the site of injury. The objective of this study was, therefore, to assess the effect of this mimetic on the recovery process after peripheral nerve injury in a rat sciatic nerve crush model. We used a quantitative biomechanical method to assess the effect of RGTA's on neural adhesions. Additionally, RGTA's were tested for their influence on the regenerative capacity of axons by using neurophysiology (MNG) and footprint analysis.

Methods

Animal Preparation

The experimental protocol was approved by the Animal Experiments Committee according to the national Experiments on Animals Act, and adhered to the rules laid down in this national law, which serves to implement the “Guidelines on the Protection of Experimental Animals” established by the Council of Europe in Directive 86/609/EC (1986). Animals were housed under standard conditions of light and accommodation, and allowed to become accustomed to laboratory conditions for 1 week before the start of the experiment. They were fed a standard laboratory diet (Hope Farms) with food and water freely available.

Surgical Procedure

A total of 32 female Wistar rats (Harlan Netherlands B.V.), each weighing ~200 g, were randomly allocated to RGTA treatment or sham treatment in a blinded design. Rats were anesthetized using isoflurane (Rhodia Organique Fine, Limited) in a mixture of oxygen and nitrous oxide. In each animal, the right sciatic nerve was exposed through a gluteal muscle-splitting incision. The nerve was mobilized in the midhigh under magnification. Prior to crush injury, a uniform segment of 10 mm was marked by epineurial sutures 10.0 Ethilon (Ethicon, Inc.). To perform a standardized nerve crush injury, 5 mm of this segment was crushed for 30 seconds by using a surgical needle holder, applying a fixed force of 57.5 N. Following local treatment with RGTA or saline, the muscle septum and skin incisions were closed with 5.0 Vicryl sutures (Ethicon, Inc.).

Pullout Force Measurements

To evaluate the degree of nerve tethering to surrounding tissue, the force required to break adhesions between the marked nerve segment and its surrounding tissue was measured, as described elsewhere. Six weeks after surgery, 16 of the 32 animals were anesthetized by inhalation of isoflurane in a mixture of oxygen and nitrous oxide, and subsequently killed by cervical dislocation. Sciatic nerves were dissected proximally and distally from the nerve segment marked in the initial operation. Each distal nerve end was then transected at the level of the distal epineural suture (labeled X2 in Fig. 1). The proximal nerve end was transected 10 mm proximal from the proximal epineural suture (labeled X1 in Fig. 1). At this point, the standardized nerve segment was only attached to its surrounding tissue.

Immobilization of the rat leg was accomplished by pinning down the upper hind portion of the leg with 19-gauge needles (Sterican, B. Braun Melsungen AG). The proximal end of the nerve segment was held tightly with a stiff vascular clamp and interconnected to a force transducer with a range of 0–500 g (model #52-9503: Harvard Apparatus, Inc.) by using a silver wire suture. A pulley was used to align the course of the sciatic nerve with the pull direction. The force transducer was connected to a motorized drive with a constant extension rate of 29 mm/minute. The force required to pull the nerve segment out of its tissue bed was recorded.

Magnetoneurography Studies

Neurophysiological measurements were performed using MNG, which has proven to be a valuable tool to quantify peripheral nerve regeneration in rabbit and rat models. The MNG measurements were performed 5 weeks after nerve crush injury, according to the ex vivo MNG technique as described earlier. In short, 16 animals were anesthetized and the sciatic nerve was exposed from its origin in the lumbar spine to the trifurcation at knee level distally, to acquire a segment of maximum length. To prevent axon leakage, the proximal part as well as the 3 branches of the sciatic nerve were ligated with a 6.0 suture and transected proximally and distally to the knots, respectively. The MNG recording chamber was filled with a buffer solution (Ringer's lactate containing glucose, 1 g/L) and kept at 21 ± 0.1°C. The nerve was guided through the recording sensor coils and the stimulation cuff, and stretched to in vivo length by clamping the 2 ligation sutures in the recording chamber (Fig. 2). The distal nerve end was stimulated with a biphasic constant current pulse of 50 μsec delivered by 2 isolated stimulus units (DS3, Digitimer Ltd.), connected in parallel. To guarantee supramaximal stimulation, the stimulator was ultimately set to 1.4 times the strength of the lowest current that produced a maximal signal.

Right (surgically treated) and left (control) sciatic nerves were both measured. In the recording setting of the right nerve, the stimulation cuff cathode was positioned 10 mm distal to the marking suture placed prior to the crush lesion (X2 in Fig. 1). Sensors 1 and 2 were positioned 4 mm and 14 mm proximal to the marking suture,
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respectively (Fig. 2). By applying distal stimulation and proximal recording, only nerve fibers that had regenerated across the lesion were stimulated and recorded. For the contralateral (control) nerve, similar recording settings were used, and an imaginary suture line was created at the same distance from the distal nerve end as was present in the crushed nerve.

In each recording setup, 4 consecutive batches of 256 compound nerve action currents were recorded and averaged using custom-made software (DataCorr3 written in Visual Basic for Applications for Microsoft Excel). Each signal was calibrated by means of an accompanying 1-μA calibration signal, which was recorded prior to every stimulus. Further analyses were performed using MatLab software (The MathWorks) to derive the following variables from the signal: 1) first peak amplitude; 2) peak–peak amplitude; 3) area; and 4) conduction velocity over the nerve segment between the stimulation and recording site.

The biphasic character of the signal derived from Sensor 1 enabled calculation of all of these variables. Because of the generally monophasic character of the signal recorded with Sensor 2, only the first peak amplitude and the conduction velocity were calculated for this sensor.

The neurophysiological variables were expressed as right/left ratios.

Footprint Analysis

Static footprint analysis was performed preoperatively and at Days 1, 7, 14, 17, 21, 24, 28, and 35, and in the animals in the pullout force experiment also at Day 42 postoperatively, following the procedure described elsewhere. From the digitized footprints, the distance between the first and fifth toe (known as “toe spread”) was measured. The averaged distances of 3 measurements were used to calculate the static TSF: TSF = (OTS – NTS)/NTS, where O denotes “operated on”/right foot and N means normal/left foot. A value of –0.66 was allocated to the static TSF in case no footprints were measurable due to total impairment.

The animals were meticulously examined for signs of autotomy and contracture. All animals missing part of the foot and/or showing contracture were excluded from footprint analysis.

Treatment With RGTA

In the experimental group (RGTA type OTR4120), 50 μl RGTA (0.1 mg/ml, in sterile physiological saline solution, freshly prepared) was locally administered for a period of 45 minutes postinjury. At Days 1, 4, 8, and 15 after surgery, 1 mg/kg RGTA dissolved in saline was injected intramuscularly. The sham-treated control group received only saline in an identical treatment protocol.

Statistical Analysis

The data were analyzed using SPSS version 10 (SPSS, Inc.) software. Statistical significance was accepted at probability values < 0.05. Individual statistical tests are mentioned where used.

Results

No animals in any of the groups displayed wound dehiscence or wound infection. One of the control animals in the pullout force experiment died preoperatively. One animal was excluded from MNG evaluation and static footprint analysis because of automutilation.
Pullout Force Results

The effect of RGTAs on the pullout force was assessed by means of the unpaired Student t-test, which demonstrated a significantly reduced pullout force in the RGTA group (p < 0.001; Fig. 3). The mean pullout force in the control group (7 rats) was 207 ± 14 g, and was reduced by 67% to 67 ± 9 g in the RGTA group (8 animals). Error bars indicate SEMs.

Magnetoneurography Results

Table 1 shows the results of the neurophysiological examination after 5 weeks. None of the 6 MNG outcome variables differs significantly between the RGTA and control groups (unpaired Student t-test).

Footprint Analysis

Visual assessment of the recovery pattern of static TSF with time after crush injury shows no distinct difference in functional loss between the RGTA and control groups (Fig. 4). In concordance with this finding, multivariate tests of repeated measures demonstrate no significant difference between the 2 recovery patterns (p = 0.698).

Discussion

Our results demonstrate that RGTAs significantly reduce adhesions after peripheral nerve crush injury. Neuropathological data show no enhanced peripheral nerve regeneration in RGTA-treated animals. The RGTA do not seem to impede axonal regeneration or functional recovery either.

Extraneural Scarring

This study revealed that nerve adherence to surrounding tissue was reduced by RGTAs. The pullout force of uninjured nerves was ~ 40 g (our unpublished data). This diminishing effect of RGTAs on extraneural scarring has not been reported earlier. In vitro, RGTAs induced a decrease of Type III collagen production by different cultured cells. Biopsy samples of intestinal tissue obtained in patients with Crohn disease that are incubated with RGTA ex vivo showed direct diminution of Type III collagen.

In most studies on prevention of extraneural scarring in peripheral nerve surgery, adhesions were evaluated by a description of histological findings, or scored based on the difficulties of surgical dissection by using a numerical grading scheme introduced by Petersen et al. To assess the antiadhesion effect of RGTA after nerve injury, we used a biomechanical method previously described by Smit et al. The measured pullout force provides a quantitative outcome, strictly related to the connections between the nerve and its surrounding tissue. Pullout force has been used previously, in 2 slightly different experimental setups, to score neural adhesions.

Fig. 2. Schematic view of the magnetoneurographic setup, demonstrating distal stimulation and proximal recording. The cross-hatched area represents the crush lesion.

Fig. 3. Bar graph illustrating the results of peak force measurements. The y axis represents the force required to break adhesions. Error bars indicate the SEMs. POF = pullout force.

Fig. 4. Schematic view of the magnetoneurographic setup, demonstrating distal stimulation and proximal recording. The cross-hatched area represents the crush lesion.
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### TABLE 1

<table>
<thead>
<tr>
<th>Sensor &amp; Group</th>
<th>Mean ± SEM</th>
<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>1PA Sensor 1 control</td>
<td>0.45 ± 0.03</td>
<td>0.53</td>
</tr>
<tr>
<td>RGTA</td>
<td>0.48 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>PPA Sensor 1 control</td>
<td>0.51 ± 0.03</td>
<td>0.51</td>
</tr>
<tr>
<td>RGTA</td>
<td>0.54 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>CV Sensor 1 control</td>
<td>0.48 ± 0.07</td>
<td>0.39</td>
</tr>
<tr>
<td>RGTA</td>
<td>0.55 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>A Sensor 1 control</td>
<td>0.67 ± 0.04</td>
<td>0.98</td>
</tr>
<tr>
<td>RGTA</td>
<td>0.67 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>1PA Sensor 2 control</td>
<td>0.64 ± 0.13</td>
<td>0.25</td>
</tr>
<tr>
<td>RGTA</td>
<td>0.51 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>CV Sensor 2 control</td>
<td>0.69 ± 0.08</td>
<td>0.77</td>
</tr>
<tr>
<td>RGTA</td>
<td>0.72 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

* A = area of Sensor 1; CV = conduction velocity of Sensors 1 and 2; 1PA = first peak amplitude of Sensors 1 and 2; PPA = peak–peak amplitude of Sensor 1.
† Values are expressed as left (control leg)/right (treated leg) ratios.
‡ Probability values determined according to the unpaired Student t-test.

Many strategies have been followed in an attempt to reduce extraneural scarring. Several surgical techniques have been developed to create an autologous barrier, such as wrapping the nerve with a vein, dermofascial fat grafts, or muscle flaps. Foreign materials providing a physical barrier, like silicone cuffs, have been studied as well. Agents are known to exert a pharmacological effect on adhesion formation, including mitomycin C (a chemotherapeutic agent), human amniotic fluid, aprotinin (a proteinase inhibitor), and anti-transforming growth factor β. Finally, hyaluronic acid gel and a membrane that is a mixture of hyaluronic acid and carboxymethylcellulose may affect adhesion formation at a cellular and mechanical level.

Axonal Regeneration and Functional Recovery

Over the years, several experimental strategies have been proven to promote nerve regeneration and functional recovery, including the use of electrical stimulation and the application of hyaluronic acid, FK506, steroids, and anti–transforming growth factor β. The effect of RGTA on nerve regeneration has not been reported. Our MNG data showed no significant difference in conduction capacity between the RGTA and the control group. This indicates no enhancement of axonal regeneration, nor a negative effect of RGTA in a crush model. The fact that the recovery pattern of static TSF did not improve or accelerate during the 6 weeks following injury supports these findings.

We hypothesized that RGTA could enhance regeneration through a reduction of intraneural fibrosis. Furthermore, RGTA might affect regeneration through binding and protecting preexisting and newly synthesized heparin-binding growth factors. The results of our study seem to refute these hypotheses, because RGTA apparently do not positively affect regeneration as measured by TSF and MNG. However, it should be noted that our results might also be explained by the type of injury applied in our model. The crush model is widely used for studies of injuries in rat sciatic nerves. Nevertheless, one of its disadvantages is that subsequent functional recovery is generally excellent. In the present study, the static TSF had practically normalized at 6 weeks in the control group. Therefore, with little left to improve, any effect of RGTA on axonal regeneration might have been too small to be observed. When the severity of the injury is increased, for example, as in a nerve repair model, acceleration and improvement of regeneration might still be demonstrated. For that reason, we emphasize the need for further studies to investigate the effect of RGTA on peripheral nerve regeneration in a nerve transection model.

Conclusions

We have proven the effectiveness of RGTA in reducing the pullout force after nerve crush injury without a detectable effect of RGTA on axonal regeneration. We
conclude that local and systemic application of RGTAs after peripheral nerve trauma has a therapeutic effect on neural adhesions and should be further investigated for clinical use.

Disclosure

Prof. Barritault is founder and president of OTR3. None of the other authors has a financial interest in the OTR3 company.

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