Reparixin, an Inhibitor of CXCR2 Function, Attenuates Inflammatory Responses and Promotes Recovery of Function after Traumatic Lesion to the Spinal Cord

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ABSTRACT

It has been shown that the blockade of CXCR1 and CXCR2 receptors prevents ischemia/reperfusion damage in several types of vascular beds. Reparixin is a recently described inhibitor of human CXCR1/R2 and rat CXCR2 receptor activation. We applied reparixin in rats following traumatic spinal cord injury and determined therapeutic temporal and dosages windows. Treatment with reparixin significantly counteracts secondary degeneration by reducing oligodendrocyte apoptosis, migration to the injury site of neutrophils and ED-1-positive cells. The observed preservation of the white matter might also be secondary to the enhanced proliferation of NG2-positive cells. The expression of macrophage-inflammatory protein-2, tumor necrosis factor-α, interleukin (IL)-6, and IL-1β was also counteracted, and the proliferation of glial fibrially acidic protein-positive cells was markedly reduced. These effects resulted in a smaller post-traumatic cavity and in a significantly improved recovery of hind limb function. The best beneficial outcome of reparixin treatment required 7-day administration either by i.p. route (15 mg/kg) or subcutaneous infusion via osmotic pumps (10 mg/kg), reaching a steady blood level of 8 μg/ml. Methylprednisolone was used as a reference drug; such treatment reduced cytokine production but failed to affect the rate of hind limb recovery.

The tissue damage caused by spinal cord injury (SCI) may derive from two distinct phases. The early mechanical injury, which directly damages the tissue, and a secondary ischemic insult, which is accompanied by a vigorous inflammatory response, may be responsible for the widespread damage involving an extensive demyelination of the surviving axons surrounding the injury site (Gorio et al., 2002, 2005; Kwon et al., 2004). The current standard therapy consists of the administration of methylprednisolone sodium succinate (60-methylprednisolone-21-hemisuccinate sodium salt) (MPSS) within 8 h of injury (Bracken, 2002; Merola et al., 2002; Szabo, 2003). However, it seems that MPSS offers only a limited benefit (Short et al., 2000). The potential explanation of this may be that MPSS treatment has beneficial effects by reducing proinflammatory cytokines (Fu and Saporta, 2005) and also less positive effects by suppressing trophic factors, such as nerve growth factor and glial-derived neurotrophic factor (Nakashima et al., 2004).

Several mediators and cell types of the inflammatory response are involved in the cascade of events triggered by ischemia and reperfusion (Gilmont et al., 1996; Willerson, 1997; Souza et al., 2002; Merchant et al., 2003; Sroga et al., 2003). Among the cell types participating in such tissue injury, neutrophils play a major role (Willerson, 1997). Thus, the development of pharmacological strategies aimed at the limitation or inhibition of neutrophil accumulation and activation at site of injury would represent a major advancement for the attenuation of secondary degeneration after SCI. CXC-ELR+ chemokines are very potent and effective activators of neutrophils (Baggiolini et al., 1995) that bind to cell surface CXCR1 and CXCR2 receptors. The blockade of these receptors prevents ischemia/reperfusion injury in several...
types of vascular beds (Boyle et al., 1999; Miura et al., 2001; Souza et al., 2004). It has recently been identified and characterized that reparixin \( R(\text{II}) - 2(4\text{-isobuthylphenyl})\text{propiony}1\text{methylanesulphonamide} \) (formerly repertaxin) is a new small-molecule inhibitor of human CXCR1/R2 and rat CXCR2 receptor activation. Structural and biochemical data are consistent with a noncompetitive allosteric mode of interaction between CXCL8 receptors and reparixin l-lysine salt, which by blocking CXCR1 and CXCR2 in an inactive conformation prevents the activated receptor-induced intracellular signal transduction cascade and cell response. This mechanism of action is unprecedented in the pharmacological modulation of G-protein coupled receptors (Bertini et al., 2004). Reparixin was proved to be efficacious in preventing neutrophil infiltration, tissue damage, and organ dysfunction in several experimental models of ischemia/reperfusion injury and organ transplantation (Bertini et al., 2004; Cugini et al., 2005; Garau et al., 2005). Moreover, by inhibiting neutrophil recruitment into reperfused organ, reparixin was also demonstrated to block cytokine production and prevent lethality of rats exposed to intestine postischemic injury (Souza et al., 2004). This clear demonstration of a marked protection in ischemia/reperfusion injury by reparixin lays the basis for the verification of reparixin activity in the attenuation of secondary degeneration triggered by traumatic SCI. It will be shown that reparixin administration attenuates the progressive secondary degeneration of the basis for the verification of reparixin activity in the attenuation of secondary degeneration triggered by traumatic SCI. It will be shown that reparixin administration attenuates the progressive secondary degeneration of the spinal cord by markedly reducing the expression of inflammatory cytokines and the infiltration into the site of injury of neutrophils and ED-1-positive mononucleated cells. As a consequence, the drug treatment attenuates the progressive secondary degeneration with a reduction of the lesion size and increases the extent of spared myelinated axons that surround the site of injury. On the other hand, it will be shown that reparixin must be administrated for, at least, 7 days to obtain a sustained recovery of hind limb motor and sensory function.

Materials and Methods

Animals. Adult Sprague-Dawley rats weighing 240 to 260 g were used. Animals were kept in our animal facilities under standard housing conditions (22 ± 2°C, 65% humidity, and lights from 6:00 AM to 8:00 PM). A standard dry diet and water were available ad libitum. All experimental protocols were approved by the Review Committee of the University of Milan and Warren Institute and met the Italian guidelines for laboratory animals, which conform to the European Communities Directive of November 1986 (86/609/EEC) as well as the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Spinal Cord Injury with UTS Impactor and Drug Treatment. The traumatic SCI was performed by means of the UTS impactor. The UTS impactor consists of a small impacting device that can be easily put over the surgical table on which the animal has been prepared. This device is connected to a console containing the controls of the impactor. The console is linked to a personal computer that records and manages the data. The core of the impactor is a metallic rod, with a flat end of 2.3-mm diameter, that is driven to the palmar surface. The response was rated as follows: 1 (no movement at 37°C was a pumping rate of 10 μl/h, a reservoir volume of 2 ml, and a duration of 7 days. The osmotic pump system guided by an optical encoder connected to a specific electronic circuit. This circuit provided an additional electric current and a force to the coil, capable of annihilating the weight of the sledge, that remained suspended. Before applying force to the spinal cord, it is necessary to place the rod at the desired height over the spinal cord of the animal. This is achieved by another optical encoder and by two infrared transmitters fixed on the frame of the magnet. Two infrared phototransistors on the sledge act as receivers that read the position. This system allows precise positioning of the sledge and thus of the impacting rod (error = ± 0.05 mm) over the target. The console carries control buttons for force and time settings. Force can vary from 0.01 to 2.0 newtons. Time can vary from 0.1 to 50 s. (Gorio et al., 2002). The impounding piston is usually positioned 1 mm above the exposed cord at T4 and set for an excursion of 3 mm. A force of 1 newton for 1 s was applied, which was followed by an automatic return of the impactor rod. Animals were anesthetized by i.p. injection (10 ml/kg) of 4% chloral hydrate (Sigma-Aldrich, St. Louis, MO) and laid over a mat kept at the temperature of 38°C; before awakening, they were treated with buprenorphine (0.03 mg/kg) (Sigma-Aldrich) for pain and penicillin G (10,000 U/kg; Sigma-Aldrich) as antimicrobial agent. A laminectomy of the T4 vertebral level was performed on anesthetized rats. The size of the laminectomy (approximately 3 mm in diameter) was consistent between animals to allow room for the impactor tip. Care was taken not to remove the lateral part of the vertebrae at the site of laminectomy to maintain column stability. The lateral processes of T4 and T6, vertebrae were cleared of muscle to allow for stabilization of the vertebral column using forceps attached to the impactor-clamping platform, and a contusion injury was induced on the exposed cord using the impactor device. The muscle was sutured with 5-0 chromic gut, and the skin was closed with 7-mm wound clips. Each experimental group was constituted by at least 18 animals. After SCI, rats were housed two per cage and had manual bladder evacuation three times daily. Reparixin (Dompé Pharma s.p.a., L’Aquila, Italy) salified with L-lysine was dissolved in saline and administered daily for 7 days at the doses indicated under Results. The 1st day treatment was a slow i.v. injection, performed within 30 min after injury, followed by s.c. injections every 2 h up to 6 h after SCI, and then reparixin was administered twice daily at 9:00 AM and 5:00 PM. In contrast, after the first i.v. injection, reparixin was administered by means of an osmotic pump with flow modulator in place (ALZET, Cupertino, CA). The pump performance at 37°C was a pumping rate of 10 μl/h, a reservoir volume of 2 ml, and a duration of 7 days. The osmotic pump without catheter was placed under the dorsal skin within 30 min after SCI at the lumbar sacral level and released the drug at the desired constant rate as explained under Results. Methylprednisolone sodium succinate (Sigma-Aldrich) was administered at a dose of 30 mg/kg i.p. injection. It is noteworthy that a single dose of 60 mg/kg and repeated daily treatment (three times) with 30 mg/kg proved to be lethal, killing all of the SCI-lesioned animals (n = 7) within the 1st week. Each experimental group was constituted by at least seven animals.

Behavioral Testing. All outcome measures were assessed in blinded fashion by four independent investigators and then averaged. Neurological function was evaluated at the first 24 h after injury and then twice a week. The methods used are well known in the field of behavioral evaluation of recovery of function after SCI. Locomotor function and hind limb recovery after contusion were evaluated with the open-field test according to the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (Basso et al., 1995). Alldynia-like responses in unaffected forepaw were assessed by means of standard hot-plate test and cold stimulation. For hot-plate testing, rats were placed on hot-plate, and the latency to licking was measured. Nonresponders were removed after 60 s. The response to cold stimulation was tested by the application of ethyl chloride spray to the palmar surface. The response was rated as follows: 1 (no
response), 2 (brief withdrawal with licking), and 3 (vocalization, withdrawal with licking, and aversion). One week before the lesion operation, animals were trained to walk on a 1-m long horizontal ladder with metal rungs elevated 30 cm from the ground. A testing session consisted of three ladder passes, and performance was captured using a digital video camera (DICAM 200, 60 fields/s; α Instruments, Granger, IN). The analysis involved counting the number of errors in placing of the right hind limb and averaging errors from the three passes for each animal. A defined 10-bar sector was chosen for analysis. To prevent habituation to a fixed bar distance, the bars in this sector were placed irregularly (1–3-cm spacing) and were changed for each testing session. The performance of each animal was monitored weekly from the 14th day until up to 5 weeks after surgery. Animals were excluded from behavioral testing if they were not able to walk on the horizontal ladder (more than eight mistakes per trial). A total of nine animals per group were used.

Histology and Immunocytochemistry. At the end of the experimental period, animals were anesthetized by inhalation of halothane and then fixed by a 4% paraformaldehyde in isotonic phosphate-buffered saline at pH 7.4 via transcardial perfusion. The spinal cord encompassing the injury site was postfixed with the same phosphate-buffered saline at pH 7.4 via transcardial perfusion. The thane and then fixed by a 4% paraformaldehyde in isotonic phosphate buffer solution, pH 7.4. Paraffin wax sections of 5 μm were stained with Harris hematoxylin and eosin. Neutrophils were identified in sections at 3 mm caudal from lesion epicenter by means of the naphthol AS-D chloroacetate technique for esterase. The substrate was dissolved in dimethyl sulfoxide/Triton X-100 (9:1; v/v), and then pararosaniline hydrochloride in saline buffer solution was added, as described elsewhere (Schlayer et al., 1988). Red-stained polymorphonuclear cells were counted in four nonconsecutive groups, randomly chosen around the lesion site of 400× histological fields. Results were expressed as number of neutrophils per histological power field (hpf).

Determination of ED-1-Positive Cells Tissue Infiltration. Cryostat sections of lesioned spinal cord were adjacent to those used for neutrophil detection (see above) and stained for ED-1. ED-1-positive cells were quantitatively evaluated as described previously for neutrophils. Results were expressed as the number of ED-1-positive cells per hpf.

Oligodendrocytes Apoptosis. Apoptosis of oligodendrocytes within the fasciulus cuneatus was determined 10 days after SCI by using the terminal deoxynucleotidyltransferase-mediated dUDP end labeling (TUNEL) methodology using 10-μm-thick sections obtained at a distance of 3.5 mm rostrally to the center of the impact site. In brief, sections were deparaffinized and then treated with proteinase K (20 μg/ml in 10 mM Tris-HCl, pH 7.6, for 15 min at room temperature), blocked in 3% H2O2 in methanol for 10 min, permeabilized for 2 min in 0.1% Triton X-100/sodium citrate at 4°C, and treated with TUNEL reaction mixture according to the manufacturer's protocol (In Situ Cell Death Detection kit; Roche Diagnostics, Basel, Switzerland). Positive neurons were identified after development for 15 min in diaminobenzidine, dehydration, and application of coverslips. Sections obtained from treated and control animals were examined by using light microscopy, and the total number of TUNEL-positive cells was determined by an observer blinded to the treatment.

Cytokine ELISA. At the indicated times, spinal cord tissue was homogenized and then sonicated (Branson Sonifier 250; Branson Ultrasonics Corporation, Danbury, CT) in a 7.4 pH buffer solution containing 10 mM Tris, 0.032 mM sucrose, 5 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The processing was performed at 4°C. Proteins were determined by Lowry-Ciocalteau technique.

The tissue concentration of inflammatory cytokines MIP-2, TNF-α, IL-6, and IL-1β were assayed at the site of injury by means of specific ELISA kits (Biosource Europe SA, Nivelles, Belgium) following the manufacturer’s instructions. At least six animals per group were included for analysis.

Statistical Analysis. Data were expressed as the mean ± S.E.M. Multiple group comparisons were made by ANOVA with a post hoc Tukey test. Two-tailed Student’s t test was used to compare the recovery of hind limb function and sensorimotor coordination after reparixin treatment with control. The analyses were performed using Prism 3.0 software (GraphPad Software, Inc., San Diego, CA). Statistical significance was accepted for a P < 0.05.

Results

Evaluation of Site of Injury and Oligodendrocyte Apoptosis. The histological examination of the injury site (Fig. 1, A–C) shows that sparing of the white matter was enhanced by reparixin treatment. The quantitative evaluation of the percentage of spared tissue at site of injury, 4 weeks after SCI, shows that the tissue degeneration (cavity size) was greatly reduced by 15 mg/kg reparixin treatment for 7 days (first treatment via i.v. route within 30 min after SCI followed by s.c. repeated administrations up to 6 h after SCI, and twice a day s.c., thereafter) and was unaffected when 30 mg/kg methylprednisolone was applied as a single i.p. injection within 30 min after SCI (Table 1). Furthermore,
Results

Percentage of spared tissue at 4 weeks after SCI. The sparing white matter is greater in reparixin-treated rats, as also shown in B and C. B, GFAP-positive cells and processes in the spared white matter. Staining with anti-GFAP shows a much greater glia reactivity in the lesioned cord of SAL-treated animals compared with REP-treated rats. Magnification bars are indicated. C, FluoroMyelin staining of the spared white matter. The better preservation of the myelin fibers in the lesioned cord of REP-treated rats is clearly shown. Magnification bars are indicated.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesion Epicenter</th>
<th>Throughout T3</th>
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<tbody>
<tr>
<td>Saline</td>
<td>39.8 ± 3.9</td>
<td>46.6 ± 3.1</td>
</tr>
<tr>
<td>Reparixin</td>
<td>48.9 ± 3.0**</td>
<td>58.2 ± 2.9**</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>41.4 ± 3.2</td>
<td>47.2 ± 3.6</td>
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** P < 0.01 compared to saline and methylprednisolone treatments.

Post-traumatic gliosis and scar formation were reduced by reparixin treatment. In saline-treated rats, GFAP-positive cells and processes were diffused throughout the spared white matter at spinal cord injury site; the extent of such positivity was greatly attenuated in reparixin-treated animals (Fig. 1B). The enhanced sparing and reduced gliosis of the white matter promoted by reparixin was corroborated by the staining with FluoroMyelin (Fig. 1C), which shows a good preservation of myelinated axons at the site of injury. We have previously shown that NG2 immunoreactivity in the spinal cord of unoperated or sham-operated and spinal cord-lesioned rats was comparable (Vitellaro-Zuccarello et al., 2007). At high magnifications, NG2 immunoreactivity appeared as puncta and filaments (Fig. 2) that were far more numerous in reparixin-treated rats. The confocal microscopy of the spared white matter showed that NG2-positive cells are more likely packed and more ramified after reparixin treatment. Double labeling experiments showed that NG2 immunostaining was performed in the ventral white matter. The results show that NG2-positive immunostaining was increased by reparixin, whereas GFAP-staining was quantitatively decreased (Fig. 2, C and D). No colocalization of NG2 and GFAP or ED-1 was ever observed in reparixin treatments (data not shown). Inflammatory cell penetration of the injury site was greatly reduced by reparixin. The number of cells positive to ED-1 antibodies at injury site was quantitatively evaluated 7 days after SCI (Fig. 3, A–C).

In keeping with the mechanism of action of the compound, treatment with reparixin also dramatically reduced neutrophil infiltration into the injury site (Fig. 4, A–C). Neutrophil recruitment was evaluated 24 h after lesioning, because the peak of neutrophil infiltration was reached at this time after SCI (Schnell et al., 1999). Reparixin treatment inhibited the number of recruited neutrophils by 90% as shown by histopathologic analysis (Fig. 4C).

TUNEL labeling in the fasciculus cuneatus 3.5 mm rostral to the lesion epicenter revealed a mean number of TUNEL-positive cells of 14.1 ± 2.1 for saline-treated animals, whereas for animal given reparixin (15 mg/kg × 7 days), the number of TUNEL-positive cells was markedly reduced to 2.1 ± 1.3 (n = 8 each group; P < 0.001).

Tissue Cytokine Concentrations. Spinal cord concentrations of inflammatory cytokines determined by ELISA within the lesion epicenter at 1, 2, or 7 days after SCI (Fig. 5) increased in a time-dependent fashion following injury, with MIP-2 (a specific CXCR2 agonist) augmentation occurring mainly within the 1st day. Such a high tissue concentration of MIP-2 was maintained up to 7 days. TNF-α peaked by 2 days and then decreased slightly but remained elevated at much higher levels compared with control laminectomy specimens for at least 7 days after injury. Both reparixin and MPSS administration prevented these increases (Fig. 5). MIP-2 and TNF-α are recruitment signals for leukocytes, and histological evaluation up to day 7 confirmed the almost complete absence of these inflammatory cells, reinforcing the observation that secondary injury would also be reduced. IL-6 also peaked within 2 days but then decreased to a much lower level. Both reparixin and MPSS greatly counteracted the maximal IL-6 levels that were maintained close to control. The post-traumatic increase of IL-1β occurred in the 1st day and was counteracted by both agents.

MIP-2 and TNF-α increased again to 39 and 99 pg/ml, respectively, at the 7th day after spinal cord injury when reparixin treatment was performed for the shorter period of treatment (up to 3 days after SCI). These values were comparable to saline treatment. In contrast, IL-6 and IL-1β remained low (data not shown).

Recovery of Hind Limb Function. Open-field locomotor testing indicated that treatment with reparixin improved recovery of hind limb function for up to 60 days. The beneficial effects of the 7-day treatment with reparixin (15 mg/kg), administered i.v. within 30 min of lesioning and s.c. every 2 h up to 6 h after SCI and then s.c. twice a day for the remaining days, were obvious between 4 and 8 days after SCI and reached a plateau between 12 and 20 days (Fig. 6A). When these treatments were performed for 1 or 3 days, the promoting effect of reparixin was transient, with a significant improvement between days 4 and 7 that did not last (Fig. 6B). Lower doses of reparixin (5 and 10 mg/kg) administered as...
described above for 7 days were less effective and failed to promote any significant improvement in the recovery of hind limb function (data not shown). Methylprednisolone treatment resulted in an early transient improvement of locomotor recovery; after 10 days, no difference with saline treatment was observed. The sensorimotor deficit caused by SCI was assessed in nine animals by means of the horizontal ladder test. We observed a progressive worsening with saline treatment that was significantly counteracted by reparixin treatment, particularly beginning at 3 weeks after injury (Fig. 6C).

The requirement of multiple doses of reparixin treatment to promote functional recovery after SCI in rats suggests that administration by continuous infusion could be the optimal schedule of treatment, in view of a potential clinical therapeutic use of the compound. To assess the efficacy of reparixin administered by continuous infusion on functional recovery after SCI, the compound (15 mg/kg) was administered i.v. within 60 min after injury followed by continuous s.c. infusion up to the 7th day. The rate used was 10 mg/h/kg with the aim of achieving a plasma steady-state concentration \( C_{ss} \) of 8 \( \mu \)g/ml (30 ng/ml free drug taking into account binding to plasma proteins) (Cavalieri et al., 2005), a plasma \( C_{ss} \) comparable to the \( C_{ss} \) achieved in human volunteers. Reparixin administration by s.c. infusion significantly induced the rate of hind limb functional recovery up to 60 days after SCI (Fig. 7). The lower rate of infusion of 5 mg/h/kg reparixin, preceded by 10 mg/kg i.v., also promoted a functional recovery significantly superior to that of saline. However, reducing the infused rate of reparixin to 2.5 mg/h/kg failed to stimulate any recovery (data not shown). When drug administration by pumps was only maintained for 4 days, there was no drug-mediated improvement of hind limb function (data not shown).

**MPSS Administration Did Not Affect the Rate of Hind Limb Recovery and Was Identical to Saline (Data Not Shown).** Both hot-plate test and cold stimulation did not reveal significant changes in forepaw sensitivity in saline- or reparixin-treated spinal cord-lesioned rats (data not shown).

**Discussion**

In this study on experimental spinal cord injury in the rat, we showed that reparixin, a noncompetitive allosteric low molecular weight inhibitor of human CXCR1 and CXCR2 and rat CXCR2, promoted recovery of hind limb function and attenuates secondary degeneration, oligodendrocyte apoptosis, production of inflammatory cytokines, and inflammatory cell infiltration at the site of injury. The enhanced motor function recovery of reparixin-treated rats was associated with improved preservation of the ventrolateral white matter. The sparing of the ventrolateral funiculus is strongly related to functional recovery (Ballermann and Fouad, 2006).

The complex summation of primary and secondary degeneration events determines the extent of tissue damage, which
normally extends much further than the limits of the mechanical impact. The attenuation of cellular and molecular abnormalities underlying the secondary injury may result then in a dramatic improvement of recovery from SCI-derived disability. It is well known that more than 90% of the traumatic injuries to the cord is not complete and that a portion of the cord axons is spared. Unfortunately, the surviving axons are unable to conduct the electric impulse. By limiting the progression of secondary injury, it should be possible to preserve the excitability of spared axons and reduce the extent of cavity formation. We have previously reported that acute administration of erythropoietin can attenuate the effects of acute SCI and accelerate neurological recovery after injury in the rat. Most of the difference in the rate of recovery were established within the first 48 to 72 h (Gorio et al., 2002). Such an action was accompanied by an attenuation of secondary injury and reduced inflammatory reaction to SCI as indicated by normalization of TNF-α, MIP-2, IL-1β, and IL-6 production and counteraction of cellular infiltration and gliosis at the site of injury (Gorio et al., 2005). A comparable outcome and a similar pattern of effects were observed in this study when spinal cord-injured rats were treated with reparixin. Treatment with reparixin had to be sustained for at least 1 week; shorter periods of treatments yielded only transitory improvements and failed to promote a stable recovery of function, even when the drug blood levels were maintained constant by means of an osmotic mini-pump. The effect was well sustained, and the recovery of hind limb function was even slightly better at 60 days after injury than at 4 weeks (Fig. 7). Reparixin exhibits a very short elimination half-life in rats (0.5 h) compared with dogs (10 h), for example. Thus, it is likely that metabolites play a role with respect to efficacy in the rat SCI model, or this could explain why prolonged dosing with osmotic mini-pumps gave the most favorable outcome. Reparixin is a noncompetitive allosteric blocker of human CXCR1/R2, as well as rat CXCR2 receptors, inhibiting intracellular signal mediators without affecting receptor binding (Bertini et al., 2004; Souza et al., 2004). Reparixin was proved to be efficacious in several models of ischemia-reperfusion injury by blocking neutrophil infiltration, tissue damage, and organ dysfunction (Bertini et al., 2004, Garau et al., 2005; Cugini et al., 2005). The compound efficacy had also been shown in a model of severe ischemia-reperfusion injury of the rat intestine (Souza et al., 2004), where the reduced damage was correlated to the inhibition of cytokine production. Indeed, intestine severe ischemia-reperfusion injury is accompanied by a marked release of pro-inflammatory cytokines, and systemic concentrations of TNF-α seem to be the best correlation of lethality in this condition (Souza et al., 2001, 2002). Reparixin treatment was associated with a complete protection from lethality induced by intestine reperfusion, and the protective effect of the compound was paralleled by the inhibition of TNF-α production and neutrophil infiltration (Souza et al., 2004), being that these neutrophils are essential for TNF-α production (Souza et al., 2001, 2002).

A complicated interplay of proinflammatory, anti-inflammatory molecules, and migratory cells also mediates the pathophysiology of the spinal cord following injury. Within the first 12 h, neutrophils first appear, with lymphocytes and microglia then mobilized to the injury site within 24 to 48 h.
Among the proinflammatory cytokines, much interest has been focused on MIP-2, TNF-α, IL-1β, and IL-6, which are key mediators of inflammation following central nervous system lesions (Merrill et al., 1996). Glial cells are a primary target of cytokines and are activated in response to many cytokines including TNF-α and IL-1 (Raivich et al., 1999). This activation can trigger further release of cytokines that might affect local inflammatory responses and neuronal survival. Giulian et al. (1993) suggested that IL-1 might promote neurotoxin release from glia and that TNF-α can induce...
apoptosis directly through the activation of Fas receptors (Park et al., 1998). MIP-2 is a primary chemotactic and activating factor for neutrophils. Overproduction of MIP-2 into injured spinal cord was associated with neutrophil infiltration, indicating a key role played by this mediator in inflammatory cell recruitment induced by SCI. In keeping with this suggestion, reparixin treatment completely prevented neutrophil infiltration into injured spinal cord. Likewise, reparixin administration strongly reduced ED-1-positive cell invasion, suggesting that neutrophil infiltration induced by SCI could play an important role in subsequent mononuclear cell recruitment. In agreement with this hypothesis, the phagocytic activity of neutrophils plays an important role in damaged tissue. Moreover, activated neutrophils are also known to produce an array of inflammatory mediators, including cytokines and chemokines (Chertov et al., 2000; Scapini et al., 2000), to regulate the cascade of events leading to TNF-α production (Souza et al., 2001, 2002). In addition, the increase of proinflammatory cytokines occurring after traumatic spinal cord injury has been successfully counteracted by 7 days of reparixin treatment. The inhibitory effect of reparixin on IL-1β and IL-6 was already observed at day 1 after SCI further sustaining a key role of infiltrated neutrophils in the production of cytokines observed after traumatic SCI. On the other hand, reparixin treatment reduced MIP-2 and TNF-α production starting from day 2 after SCI, indicating that resident cells and infiltrated mononuclear cells could be the main source of these factors. A similar effect on cytokines was also observed with MPSS, which, however, did not affect injury and did not promote recovery of hind limb function. High-dose glucocorticoid administration has been shown not only to inhibit proinflammatory cytokines (Sapolsky et al., 1985; Lankhorst et al., 2000; Diem et al., 2003) but also neuroprotective growth factors, such as glial-derived neurotrophic factor (Nakashima et al., 2004), brain-derived neurotrophic factor, and neurotrophin-3 (Hayashi et al., 2000). Thus, it is conceivable that this nonselective suppressive action by MPSS may affect the possible positive outcome deriving from the inhibition of proinflammatory cytokine production. On the contrary, the more selective action of reparixin results in the promotion of hind limb functional recovery after traumatic injury to the spinal cord. Preliminary results suggest that reparixin treatment increases neurotrophin-3 and brain-derived neurotrophic factor significantly at the site of traumatic injury in the spinal cord (unpublished results). In addition, we observed that shorter periods of reparixin treatment yielded only a transitory improvement and failed to promote a stable recovery of function. Such a loss of effects correlated with a rebound increase of MIP-2 and TNF-α to levels comparable to saline treatment, suggesting a return of the neuroinflammatory reaction. These data indicate that the suppression of these two proinflammatory cytokines may represent the key mechanism by which reparixin is capable of promoting recovery of function.

In traumatic models of SCI, ventral white matter is progressively involved by the spreading of chronic demyelination, macrophages/microglia infiltration, and reactive astrogliosis (Grossman et al., 2001). White matter lesions may be repopulated by new oligodendrocytes derived from NG2-expressing oligodendrocyte precursors oligodendrocyte precursors (Zhang et al., 2005). Here, we report that reparixin treatment enhances proliferation of oligodendrocyte precursors; this effect together with the reduction of apoptosis may have contributed to the white matter sparing.

In conclusion, this study shows that the 7-day blockade of CXCR2 with reparixin leads to an almost complete inhibition of neutrophil infiltration into injured spinal cord. Such an effect is accompanied by down-regulation of several proinflammatory cytokines, markedly reduced oligodendrocyte apoptosis, inhibition of mononuclear inflammatory cell infiltration and gliosis, and significant improvement in hind limb recovery of function.

References


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