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Olfactory ensheathing glia graft in combination with FK506 administration promote repair after spinal cord injury

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The aim of this study was to determine whether a combination of olfactory ensheathing cell (OEC) graft with the administration of FK506, two experimental approaches that have been previously reported to exert protective/regenerative effects after spinal cord injury, promotes synergic restorative effects after complete or partial spinal cord injuries. In partial spinal cord injury, combination of an OEC graft and FK506 reduced functional deficits evaluated by the BBB score, motor-evoked potentials (MEPs) and H reflex tests, diminished cavitation, astrogliosis and increased sparing/regeneration of raphespinal fibers compared to untreated and single-treatment groups of rats. After complete spinal cord transection, the combined treatment significantly improved functional outcomes, promoted axonal regeneration caudal to the lesion, and diminished astrogliosis compared only to non-transplanted animals. Slightly, but non-significant, better functional and histological results were found in OEC-grafted animals treated with FK506 than in those given saline after spinal cord transection. Nevertheless, the combined treatment increased the percentage of rats that recovered MEPs and promoted a significant reduction in astrogliosis. In conclusion, this study demonstrates that OEC grafts combined with FK506 promote additive repair of spinal cord injuries to those exerted by single treatments, the effect being more remarkable when the spinal cord is partially lesioned.

Keywords: Ensheathing glia; FK506; H reflex; Motor-evoked potentials; Regeneration; Spinal cord injury

Introduction

After spinal cord injury functional losses are due to neural degeneration and disruption of spinal axonal pathways not only as a direct consequence of the primary insult, but also because of secondary cell death events that occur hours and days after the initial trauma (Schwab and Bartholdi, 1996). For this reason, many experimental approaches highlight the importance of breaking the secondary injury processes to protect the spinal cord parenchyma, and thus, limit the loss of functions. Over the last two decades, a large number of drugs, including steroids, gangliosides, ion channel blockers, antioxidants and free radical scavengers have shown mild therapeutic effectiveness in experimental spinal cord injury (Kwon et al., 2004). However, to date only the administration of methylprednisolone has shown effectiveness in humans (Bracken, 2001), despite its use has become controversial since data from some clinical trials failed to demonstrate conclusive beneficial outcomes (Hurlbert, 2000; Bracken and Holford, 2002).

Another mechanism to promote functional recovery after spinal cord injury is by enhancing axonal regeneration. Several strategies, including blocking myelin or glial scar inhibitors, delivery of neurotrophic factors, and cell transplantation have demonstrated to induce axonal outgrowth after experimental spinal cord injury. Among them, olfactory ensheathing cell (OEC) grafts have been shown to promote neuroprotection, axonal regeneration and functional recovery after incomplete spinal cord injury (Li et al., 1998; Plant et al., 2003; Verdú et al., 2003a,b). Moreover, we have recently reported that OECs induced long distance axonal regeneration and mild recovery of locomotion and motor-evoked potentials when they are transplanted into thoracic complete spinal cord transection (López-Vales et al., 2006a).

Despite the promising effects exerted by OECs after spinal cord injury, restoration of functions lost after injury is achieved only to a limited degree. For this reason combination of OEC grafts with pharmacological therapies has been advocated. Thus, OEC transplants have been combined previously with methylprednisolone or with methylprednisolone and interleukin 10 (IL-10) after dorsal columns section or spinal cord contusion (Nash et al., 2002; Pearse et al., 2004). Although these studies showed that combined treatment resulted in higher corticospinal regeneration and more neuroprotection in comparison to injured-control animals, they failed to induce synergic effects with respect to OEC-grafted alone animals. Lack of potentiating effects may be due to the use of drugs that might interfere with the potential restorative properties of OECs. For example, we have recently found that administration

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of iNOS and COX-2 inhibitors counteracted the protective effect of an OEC transplant after photochemical partial spinal cord injury (López-Vales et al., 2006b). Methylprednisolone and IL-10 block the inflammatory response, a process that is modulated by OECs (López-Vales et al., 2004b). This fact may explain the limited effects of methylprednisolone or methylprednisolone/IL-10 when combined with OEC transplants.

FK506 is a FDA-approved immunosuppressant macrolide drug used to prevent allograft rejection in organ transplantations (Gold et al., 2004). However, it has been reported that non-immunosuppressant doses of FK506 exert neuroprotection (López-Vales et al., 2005) and axonal regeneration (Udina et al., 2002, 2003; Gold et al., 2004; Gold and Zhong, 2004) after spinal cord and peripheral nerve injuries. Although the restorative mechanisms induced by FK506 are not completely elucidated yet, it is known that non-immunosuppressant doses do not interfere with inflammation (Gold et al., 2004; López-Vales et al., 2005). Interestingly, FK506 in combination with allogenic Schwann cell transplants enhances regeneration of axons across long peripheral nerve gaps (Udina et al., 2004). Since OECs share many phenotypic characteristics with Schwann cells (Wewetzer et al., 2002), we hypothesized that FK506 could also exert synergic effects when combined with OECs after spinal cord injury. Thus, the present study was undertaken to determine whether the restorative properties of OEC grafts might be enhanced when they are combined with administration of FK506 after either complete or incomplete spinal cord injuries.

Material and methods

OEC cultures and immunopurification

Primary olfactory bulb cultures were set up from postnatal (P22–23) and adult Sprague-Dawley rats using the method described by Gudiño-Cabrera and Nieto-Sampedro (1996, 2000) with minor modifications (López-Vales et al., 2004a; Verdú et al., 2003a). Briefly, olfactory bulbs were removed, the meningeal layer was stripped off with fine forceps and the bulbs were chemically digested and mechanically dissociated. The cell mixture was recovered by centrifugation and seeded in flasks pre-coated with poly-L-lysine (10 μg/ml, Sigma, St. Louis, USA). A medium made by Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DMEM, D8900, Sigma) supplemented with 10% FCS, 2 mM glutamine, 10 U/ml penicillin, 10 mg/ml streptomycin and 50 mg/ml gentamycin, was used for seeding and expanding the OECs. After reaching confluence, OECs were detached and immunopurified by means of magnetic dynabeads M-450 (Dynal, Oslo, Norway) pre-coated with goat anti-mouse IgG and attached with mouse monoclonal 192 IgG antibodies (MAB365, Chemicon, Temecula, CA) that recognize the p75-NGF receptor. The p75-positive OECs were resuspended in freezing medium (C-6295, Sigma) and transferred to −20°C for 10 min, then to a deep freezer at −80°C. For transplantation, frozen cell vials were thawed at 37°C, washed two times by centrifugation (900 rpm, 7 min) in DMEM, and the pellet resuspended in the same medium. It has been previously demonstrated that frozen and thawed OECs show active migration and integration in the central nervous tissue (Gudiño-Cabrera and Nieto-Sampedro, 1996, 2000; Verdú et al., 2001), and are capable of extensive remyelination following transplantation into the adult rat CNS (Smith et al., 2002). Trypan blue stain testing was repeated three times to evaluate the viability of thawed OECs. Our counts showed that the percentage of thawed viable cells was about 90%.

Surgical procedure and OEC transplantation

Female adult Sprague-Dawley rats (250–300 g) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and subjected to a dorsal laminectomy at T8 vertebra. For the partial injury model, the exposed spinal cord was bathed with rose Bengal solution (1.5% in saline, Sigma) for 10 min and the excess dye was removed by double saline rinse. Spinal cord injury was induced by illuminating the dorsal surface of the spinal cord at a light intensity of 200 kLux during 2.5 min by means of a 5-mm diameter optic fiber positioned 10 mm on top of the cord which was connected to a halogen lamp equipped with a 100-W Xenophot halogen bulb. To obtain reproducible light intensity, the irradiation power at the fiber outlet was measured by means of a digital lux tester (Verdú et al., 2003b).

For complete transection, laminectomy was done to expose T8-T9 spinal cord segments, and the spinal cord was completely transected at T8 using microscissors. The rostral and caudal stumps were carefully lifted up to verify complete transection (López-Vales et al., 2006a).

Thirty minutes after injury, a suspension of OECs was stereotaxically injected with a sterile glass needle (80 μm i.d.; 100 μm o.d.) connected to a Hamilton syringe. Rats received four injections of OECs (3 μl each), two injections 1 mm cranial and two 1 mm caudal to the lesion epicenter, and at each level one injection at each side 0.5 mm lateral to the cord midline at 0.5 mm depth (López-Vales et al., 2004a, 2006a). The total number of OECs transplanted in rats subjected to photochemical injury was 180,000, and in rats with cord transection 1,500,000 cells. A higher number of OECs were transplanted into the spinal cord after transection than photochemical injury because of the higher severity of the complete lesion. The number OECs grafted was chosen in accordance with the results found in previous studies in our laboratory (García-Alías et al., 2004; López-Vales et al., 2004a, 2006a,b; Verdú et al., 2003a). After transplantation, the wound was closed and the animals were kept in a warm environment until full recovery.

Animals received FK506 (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) diluted in saline solution at an initial dose of 2.0 mg/kg intravenously given 5 min following surgery, followed by daily intraperitoneal injections of 0.2 mg/kg for 90 days, or the same protocol but using only saline (López-Vales et al., 2005). It is worth noting that the 0.2 mg/kg dose of FK506 has no immunosuppressive effects (Udina et al., 2003). Table 1 shows the experimental groups included in the study and the treatments

Table 1
Experimental groups evaluated in the present study

Lesion	Transplant	Treatment	Group
Photochemical	DMEM	Saline ($n=8$)	DMp-SS
		FK506 ($n=5$)	DMp-FK
	OEC	Saline ($n=8$)	OECp-SS
		FK506 ($n=8$)	OECp-FK
Transection	DMEM	Saline ($n=12$)	DMs-SS
		FK506 ($n=9$)	DMs-FK
	OEC	Saline ($n=9$)	OECs-SS
		FK506 ($n=9$)	OECs-FK