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Administration of low-dose FK 506 accelerates histomorphometric regeneration and functional outcomes after allograft nerve repair in a rat model

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SUMMARY. **A substantial loss of peripheral nerves requires grafts for repair. In animal experiments, the use of allografts is successful only when rejection of the transplant is prevented and nerve regeneration is improved by the administration of the immunosuppressant FK 506 used in high doses. In this study, we examined the functional and morphometric outcome after allograft transplantation of the sciatic nerve in rats at low doses of FK 506. Functional recovery and quantitative assessment of myelination were investigated in un-operated controls, in rats receiving isograft transplants without FK 506 treatment and in rats receiving allograft transplants with FK 506 treatment (0.1 mg/kg and 0.2 mg/kg per day). Walking-track analysis at 4, 8, 12 and 16 weeks post-operation revealed significant functional recovery in allograft with FK 506 (0.1 mg/kg) compared with other groups, although levels of the un-operated controls were not reached. At 16 weeks, myelination of nerve sections from FK 506 (0.1 mg/kg)-treated and un-operated animals did not differ significantly. There was significantly less effect of the 0.2 mg/kg dose than of the 0.1 mg/kg dose, both in the histomorphological outcome and in the functional outcome. These findings indicate that higher doses of FK 506 are not necessary for nerve regeneration, and low-dose administration could be acceptable for clinical settings in future.** © **2009 European Association for Cranio-Maxillo-Facial Surgery**

Keywords: **nerve transplantation, nerve reconstruction, FK 506, sciatic functional index, ankle stance angle, myelin basic protein**

INTRODUCTION

Severe nerve lesions with loss of neural tissue require surgical repair to enable nerve regeneration. The use of grafts of autologous origin should achieve optimal results but has several important disadvantages such as the sacrifice of a healthy nerve from the patient, limited supply of donor nerves and mismatch in size between nerve and grafts (*Oritguela* et al., 1987; *Rappaport* et al., 1993). Because the supply of autografts is limited, the use of allografts is a promising alternative. However, the antigenicity of the graft tissue requires almost immunosuppressive therapy to avoid rejection and to enable regeneration. In this context, the use of immunosuppressive therapy is questionable because of the secondary risks and toxic effects of long-term and high-dosage immunosuppressant drugs (*Neuhaus* et al., 1994; *Rifai* et al., 2006).

Significant improvement in nerve regeneration rates could minimize denervation changes and improve long-term functional recovery from nerve injuries: the immunosuppressive drug FK 506 has been shown to have neuroprotective and neurotrophic actions in experimental models, increasing neurite elongation and accelerating the rate of nerve regeneration in vitro and in vivo (*Lassner* et al., 1989; *G*old et al., 1995; *Wang* et al., 1997; *Katsube* et al., 1998; *Doolabh* and *Mackinnon*, 1999; *Jost* et al., 2000; *Lee* et al., 2000; *Wang* et al., 2002; *Udina* et al., 2002; *Hontanilla* et al., 2006). Thus, FK 506 could be useful, even clinically, for enhancing regeneration after surgical repair by improving the rate of axonal growth with allografts. In fact, there are reports of positive results regarding nerve regeneration in animals and humans immunosuppressed by FK 506 (*Gold* et al., 1994; *Berger and Lassner*, 1994; *Mackinnon* et al., 2001; *Yang* et al., 2003; *Martin* et al., 2005; *Song* et al., 2005; *Snyder* et al., 2006). Although previous studies found that FK 506 is maximally effective when administered in high doses (5–10 mg/kg/day) during the entire regeneration period in rat sciatic nerve models (*Wang* et al., 1997; *Udina* et al., 2004), prolonged systemic immunosuppression might not be justified for ensuring the success of nerve regeneration. It is, therefore, important to determine the optimal treatment dosage for FK 506 after allograft transplantation. In addition to supporting previous findings, this study examines the effect of low-dose FK 506 on nerve regeneration in a model more applicable to the severe peripheral-nerve injuries seen in clinical settings and more feasible to reduce the high doses of FK 506 accompanied with high rates of side-effects for a non-vital indication (*Wang* et al., 1997; *Udina* et al., 2004). Therefore, in this study choices of rat strains were made based on their nearly identical rat transplantion complexes (RTC), except for a variability in one of four haplotypes of the RT1 complex (RT1.C) (*Günther* and *Walter*, 2001). This is comparable to the necessary match of human leukocyte antigen (HLA) complexes before performing transplantations in humans. After the transplantation of kidneys between these

strains, the survival time of the organs was >100 days without immunosuppression, whereas skin grafts showed massive rejection during this time (*Stark* et al., 1979).

Therefore, the findings presented in this study have the potential to affect significantly the clinical management of severe peripheral-nerve injuries.

MATERIAL AND METHODS

All experiments were carried out in accordance with the National Institutes of Health (NIH) regulations for animal care and an animal use protocol was approved by the National Animal Care and Use Committee. Every effort was made to minimize the number of animals and their suffering.

Animal surgery and transplantation

Adult male Lewis (LEW/HanHsd; $n = 20$) and Dark Agouti rats (DA/OlaHsd; $n = 20$) were bought from a commercial dealer (Harlan Winkelmann GmbH, Borchen, Germany) and used at an age of 12 weeks. Animals were randomly assigned to one of five groups ($n = 4$ of each strain for each group). Group I rats received no treatment and served as un-operated controls. Surgical procedures were performed under deep anaesthesia (40 mg/100 g of chloral hydrate) and aseptic conditions. Rats of group II, III, IV and V underwent a gluteal muscle-splitting incision with exposure and neurolysis of the sciatic nerve on the left side. The nerve was resected over a distance of 15 mm, 3 mm proximal to its trifurcation. In group II rats, the gap was repaired by interposing a nerve segment grafted from animals of the same strain (isograft); group III, IV and V rats received nerve segments from the other strain (allograft). Segments were transplanted between the proximal and distal stumps with two or three 9-0 resorbable perineural microsutures at each end. The segment was sutured so that its fascicular and longitudinal orientation was preserved and ensured an optimal graft repair. The wound was closed by interrupted 3-0 resorbable sutures and was infiltrated with Bupivocain 0.5% for post-operative local anaesthesia. Animals of group III served as controls without further neuroregenerative adjuvant therapy. Rats of group IV received intramuscular injection of FK 506 (0.1 mg/kg/d, Astellas GmbH, Munich, Germany), rats of group V received FK 506 in doses of 0.2 mg/kg/d. Rats of group IV and V were examined and weighed weekly to adjust doses of FK 506 and to assess signs of toxicity such as paralysis or automutilation. Nerves of the contralateral limbs were preserved as intra-animal controls. Rats were given food and water ad libitum.

Histological procedure and morphometric analysis

After 16 weeks, animals were sacrificed by use of a lethal dosis of chloral hydrate. The interposed nerve segments of the transplanted side including 3 mm distal and proximal of the nerve graft, nerve segments of the control limb and that of un-operated animals were removed. Nerves were fixed in phosphate buffer (PB; pH 7.2) containing glutaraldehyde and paraformaldehyde (each 2%). After fixation and rinsing in PB, the nerve of the transplanted side was cut into three portions corresponding to the sides proximal and distal to the nerve graft and the nerve graft itself. Nerves were cryo-protected in a PB-sucrose solution, embedded in compound and cut on a cryostate into 40 μm transverse sections. After blocking with bovine serum, sections were incubated in a primary polyclonal antibody (Rabbit Anti-Myelin Basic Protein, Chemicon; 1:200) over-night, and were further developed by the use of an ABC Kit (Vector Lab. Inc.). DAB was used as a chromogen and was intensified by addition of $NiSO_4$ and $CoCL_2$ (each 2%). Sections were dehydrated and coverslipped with Entellan for light microscopic examination. Images of the sections were digitized at a magnification of 400 and analysis was performed on two randomly selected areas of 100 μm × 100 μm in black and white modus using image analysis software (Matrox Inspector version 8.0). The 100 μm sampling grid was based on statistical analysis using an optical dissector as a non-biased stereologic technique. Thresholds to distinguish in the black and white modus non-specific background myelin staining from true myelin basic protein (MBP) were given by constant software setups referring to analysis of MBP myelination rates in sections of the un-operated control group. MBP-density was calculated in percent of the ratio black pixels/total amount of black and white pixels per sampling grid.

Functional assessment

Functional nerve regeneration was evaluated by the sciatic functional index (SFI) (*De Medinaceli* et al., 1982) and the ankle stance angle (ASA) (*Lin* et al., 1996). Data for SFI were obtained such that hind feet of the rats were dipped in dark ink and the rats walked across a wooden corridor. The footprints were recorded on paper laid onto the bottom of the corridor. The distance between the third toe and heel, first and fifth toe, second and fourth toe, and the distance from the rostral tip of the foot to that of the opposite foot was measured on the experimental side [experimental side print lenght (EPL), experimental side total spreading (ETS), experimental side intermediary spreading (EIT), and experimental side distance to opposite foot (ETOF), respectively] and the contralateral normal side [normal side print lenght (NPL), normal side total spreading (NTS), normal side intermediary spreading (NIT), and normal side distance to opposite foot (NTOF), respectively]. The SFI was calculated as follows: $SFI = (ETOF - NTOF/NTOF + NPL - EPL/EPL + ETS - NTS/NTS + EIT - NIT/NIT) \times 220/4$. In general, the SFI around 0% corresponds to normal function, and an index of −100% represents total dysfunction. The ASA was measured during the midstance phase of locomotion. The midstance phase of the rat step cycle is defined as that phase in which the opposite hind limb is in its swing phase, and the entire weight of the lower body is borne on the examined hind limb just before the moment when the animal started to raise its heel and prepared to take-off (*Lin* et al., 1996). Recordings were taken with a digital video camera (DCR-TRV900E Sony Corp.) positioned in 1 m distance lateral to the walking-track corridor. The entire walk of rats was