Efectes d'una dosi contínua o discontínua d'FK506 en les reseccions nervioses severes reparades amb empelts autòlegs i al.logènics

Comparison of continuous and discontinuous FK506 administrations on autograft or allograft repair of sciatic nerve resection.
Udina E, Gold BG, Navarro X.
(Manuscrit, acceptat per publicar a Muscle Nerve)

Long term effects of discontinuous administration of FK506 on regeneration through allogeneic grafts in the sciatic nerve of the mouse (addenda)

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COMPARISON OF CONTINUOUS AND DISCONTINUOUS FK506 ADMINISTRATIONS ON AUTOGRAFT OR ALLOGRAFT REPAIR OF SCIATIC NERVE RESECTION

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ABSTRACT

FK506 is an immunosuppressant drug that also possesses neuroregenerative properties. It has been shown to enhance the rate of axonal regeneration and to improve recovery after nerve lesions. Nevertheless, prolonged immunosuppression may not be justified to assure the success of nerve regeneration. In this study we compared the effects of continuous and discontinuous FK506 treatment on regeneration and reinnervation after sciatic nerve resection repaired with autologous or allogenic grafts in the mouse. For each type of repair, one group received FK506 (5 mg/kg) for 4 months, whereas a second group was treated with FK506 at 5 mg/kg for 5 weeks followed by 3 mg/kg for 4 weeks; a control group received saline only. Functional reinnervation was assessed by non-invasive methods to determine recovery of motor, sensory and autonomic functions in the hindpaw over 4 months after operation. Morphological analysis of the regenerated nerves was performed at the termination of the study. Autografts and allografts treated with sustained FK506 (5 mg/kg) reached high levels of reinnervation and followed a course of recovery faster than controls. The numbers of myelinated fibers were also similar. Allografts without immunosuppression demonstrated a slower rate of regeneration, exhibiting lower final levels of recovery compared to other groups and containing fewer numbers of regenerating myelinated fibers. Withdrawal of immunosuppressant therapy resulted in a decline in the degree of reinnervation in all functions tested during the third month, with stabilization between the third and fourth months. The number of regenerated myelinated fibers in this group was significantly lower than in autografts. In conclusion, continuous or discontinuous FK506 administrations slightly accelerated the rate of reinnervation in autografts. In allograft repair, FK506 significantly enhanced both the rate and the degree of regeneration and recovery, but withdrawal of the drug resulted in graft rejection, a marked deterioration in function, and loss of regenerating fibers

Key words*: nerve regeneration, allograft, autograft, FK506, immunosuppression, sciatic nerve.*

INTRODUCTION

Severe nerve lesions with loss of neural tissue require surgical repair to enable nerve regeneration to occur. The use of grafts of autologous origin achieves optimal results [27] but has several important disadvantages, such as sacrifice of a healthy nerve of the patient, limited supply of donor nerves, and mismatch in size between nerve and grafts. Because the supply of autografts is limited, the use of allografts is a promising alternative (for review, see Evans et al.[8]). However, the antigenicity of the graft tissue requires immunosuppressive therapy to avoid rejection and allow regeneration. In this context, however, the use of immunosuppressive therapy is questionable due to the secondary risks and

toxic effects of long-term immunosuppressant drugs.[3,43]

FK506, an immunosuppressive drug, has been shown to possess neuroprotective and neurotrophic actions in experimental models,[11,18] increasing neurite elongation in vitro [28] and accelerating the rate of nerve regeneration in vivo.[13,14,50] Thus, FK506 may be useful clinically to enhance regeneration after surgical repair, either by improving the rate of axonal growth with autografts [6,38] or by avoiding rejection of allografts.[5,10,51] In fact, there are reports of good results in humans immunosuppressed by either FK506 or cyclosporin A after receiving long nerve allografts, with only a few exhibiting rejection.[29] There are also three reports of successful nerve regeneration in allogenic hand transplantation in patients treated with FK506.[7,23,32] Nevertheless, prolonged systemic immunosuppression may not be justified to assure the success of nerve regeneration. It is, therefore, important to determine the optimal treatment dosage for FK506 in autografts and allografts and to examine the consequences of withdrawal of the immunosuppressant drug in allografts. Whereas previous studies have found that FK506 is maximally effective when given during the entire regenerative period in the rat sciatic nerve crush model,[12] it seems less effective when treatment is delayed more than 3 days following transection,[44] with regeneration being slower and functional recovery lower than after crush lesion.[39] Besides the issue as to whether continuous administrations are beneficial or toxic, what happens with well-tolerated allografts when immunosuppression is withdrawn remains controversial.[5,35,53,55,56] In the present study, we compared the efficacy of continuous and discontinuous FK506 administration after resection of the mouse sciatic nerve repaired either with autografts or allografts.

MATERIAL AND METHODS

Surgical procedure

All surgical procedures were performed under pentobarbital anesthesia (60 mg/kg i.p.) in 6 groups of 2.5-month old female OF1 outbred mice (n = 6 - 8, see Table 1). The experimental procedures were approved by the Ethics Committee of our university and were carried out in accordance with the European Community Council Directive (86/609/EEC).

The sciatic nerve was exposed at the midthigh, transected at a fixed point (45 mm from the tip of the third digit), and a 6-mm segment of the distal stump resected to leave a gap that was repaired by interposing a 6-mm nerve segment grafted between the proximal and distal stumps with two 10-0 monofilament epineurial sutures at each end. For the autograft groups, the same segment resected was sutured preserving its fascicular and longitudinal orientation, to obtain the most ideal graft repair. In allograft groups, a nerve segment was harvested from the sciatic nerve of another OF1 mouse of a different litter than the recipient mouse. We have previously found that such allograft in an outbred strain induces a strong immune rejection during the first week, [51] similar in severity to that found when the allograft is obtained from a mouse of a different

strain. The wound was sutured and disinfected with povidone-iodine. In order to avoid autotomy after denervation, animals were pretreated with amitriptyline.[37] For each type of graft (autograft and allograft), two groups were treated with subcutaneous injections of FK506 (Fujisawa Pharmaceuticals, Osaka, Japan) in the back starting on the day of operation. The drug was diluted in saline solution and administered at a dose of 5 mg/kg. One group was given daily injections for the full 4 months of study (continuous treatment). The other group received the same dose only for the first 5 weeks after the operation; then the dose was reduced to 3 mg/kg for 4 additional weeks and, thereafter, withdrawn (discontinuous treatment). For each type of graft, an additional group of mice received only saline solution and served as controls.

Functional evaluation of regeneration

Target organ reinnervation was periodically evaluated with a battery of functional tests.[39] The sciatic nerve was stimulated percutaneously through a pair of needle electrodes at the sciatic notch and the compound muscle action potential (CMAP) recorded from plantar and gastrocnemius muscles with microneedle electrodes. For sensory nerve conduction, the electrodes were inserted in the fourth toe to record the compound nerve action potentials (CNAP) of the fourth digital nerves. Square pulses (0.01-ms duration) were applied at a voltage that gave a maximal response. The evoked CMAPs and CNAPs were amplified and displayed on a storage oscilloscope at settings appropriate to measure the amplitude and latency. Sweat gland (SG) reinnervation was evaluated by the silicone mold technique. Here, pilocarpine nitrate (5 mg/kg) was subcutaneously injected and 10 min later a silicone material (Elasticon, Kerr Co., Romulus, MI) was spread over the plantar surface of the hindpaw. The number of reactive SGs was determined by counting the sweat droplet impressions made in the mold under a dissecting microscope. Recovery of pain sensitivity was assessed by pricking with a needle at 5 distinct areas on the plantar surface of the denervated paw under a dissecting microscope. A subjective score to pinprick (PP) was assigned from no response (0), reduced or inconsistent responses (1) to normal reaction (2) in each area tested.

Functional tests of reinnervation were performed before operation to obtain baseline control values and at 14, 21, 30, 40, 50, 60, 90, and 120 days postoperation (dpo). For normalization, values obtained in each test after operation were expressed as the percentage of preoperative values for each mouse. For each functional parameter indicative of the degree of reinnervation (amplitude of CMAPs and CNAPs, number of reactive SGs, and PP score), we determined the day of the first response after denervation and the percentage of maximal recovery achieved at the end of the study. The overall functional recovery index (FRI), representing the area under the curve for the reinnervation data, was also calculated.

Morphological evaluation of nerve regeneration

At the end of the study, animals were reanesthetized, the operated nerve dissected from surrounding tissues, and a long segment (including several mm proximal and distal to the graft) was harvested. The regenerated nerves were fixed in glutaraldehyde-paraformaldehyde (3%-3%) in 0.1 M cacodylate buffer (pH 7.4, 4h, 4ºC) and cut in three pieces (corresponding to proximal, mid-graft and distal levels of the nerve). Tissue samples were postfixed in OsO_4 (2%, 2h), contrasted with uranyl acetate (2.5%), dehydrated through ethanol series, and embedded in Epon. Transverse semithin sections (0.5 µm) were stained with toluidine blue and examined by light microscopy. Images were acquired with an Olympus DP50 camera (Olympus Optical Co.,

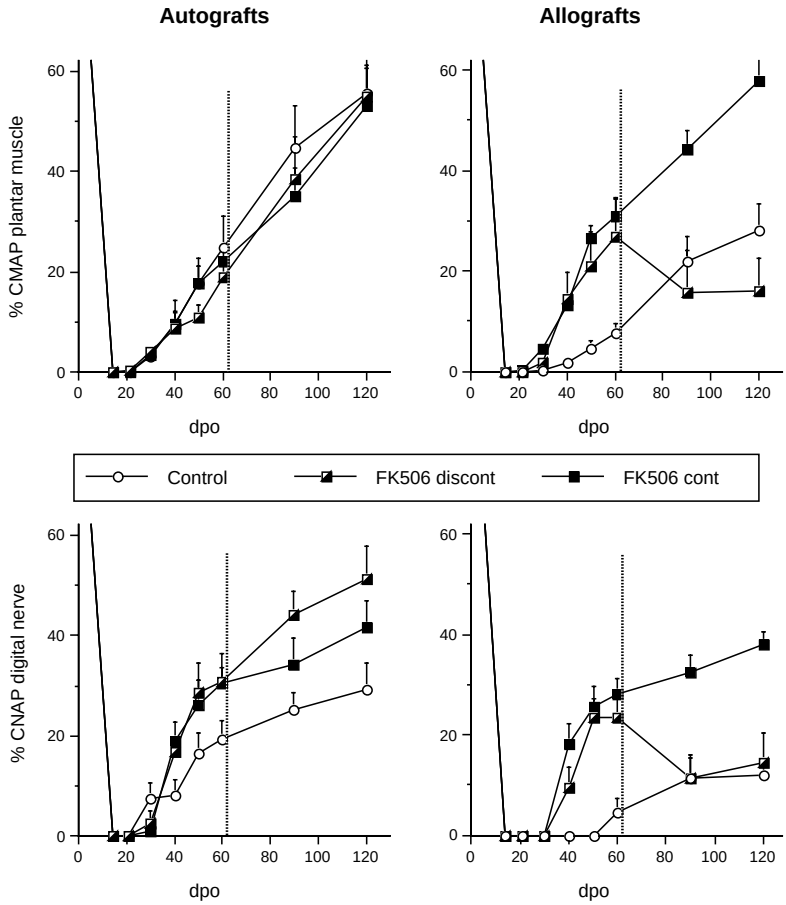

Fig. 1. Percentage of the amplitude of the CMAP of plantar muscles and the CNAP of digital nerves over time in mice with sciatic nerve resection and repair with a nerve autograft (left panel) or a nerve allograft (right panel) untreated (Control) or treated with FK506. FK506 was administered during the 4-month follow-up in groups with continuous treatment (FK506 cont), and until the time indicated by vertical dotted lines in groups with discontinuous treatment (FK506 discont)

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Hamburg, Germany) to a PowerMacintosh computer (Apple Computer, Cupertino, CA) and processed to a final magnification of 200X for measuring the cross-sectional area of the whole nerve using NIH Image software, and to 2000X for counting the number of regenerated myelinated fibers. Morphometric analysis of myelinated fibers was performed on systematically selected fields of semithin sections at the midgraft printed at 2000X. With the aid of a digitizing tablet the outer and inner profiles of myelinated fibers were marked. At least 500 myelinated nerve fibers were evaluated for each nerve. The following morphometric parameters were assessed: axon perimeter and diameter, fiber perimeter and diameter, myelin sheath thickness, and the g-ratio.were fixed in glutaraldehyde-paraformaldehyde (3%-3%) in 0.1 M cacodylate buffer (pH 7.4, 4h, 4ºC) and cut in three pieces (corresponding to proximal, mid-graft and distal levels of the nerve). Tissue samples were postfixed in OsO4 contrasted with uranyl acetate (2.5%), dehydrated through ethanol series, and embedded in Epon. Transverse semithin sections (0.5 µm) were stained with toluidine blue and examined by light microscopy. Images were acquired with an Olympus DP50 camera to a PowerMacintosh computer and processed to a final magnification of 200X for measuring the cross-sectional area of the whole nerve and to2000X for counting the number of regenerated myelinated fibers.

Statistical analysis

All data are expressed as mean ± SEM. Statistical comparisons between groups were made by ANOVA followed by a post-hoc Fisher test. Differences were considered significant at $p < 0.05$.

RESULTS

Functional results

The gastrocnemius CMAPs reappeared at 21 dpo in all groups, except in the group with untreated allograft (mean onset at 32 dpo). The final CMAP amplitude was approximately 80% of the preoperative values in autografts and allografts given continuous FK506 administration, whereas in allografts receiving discontinuous administration the amplitude declined from its peak of 61% at 60 dpo (3 days before stopping treatment) to a final amplitude of 34%. In untreated allografts, the slope of recovery was lower and the final amplitude was 68%. The reinnervation of plantar muscles, as judged from the amplitude of recorded CMAPs, exhibited a similar course of recovery in the three groups with autografts. The first CMAPs were found at 30 dpo in most mice with autograft repair, as polyphasic potentials of small amplitude and long latency. The latency shortened progressively and the amplitude increased to reach final mean values of approximately 55% with respect to preoperative baseline values (Fig. 1, Table 1). For the allografts with continuous treatment, the recovery of CMAP amplitude paralleled that of autografts, with a final value of 58%, whereas untreated allografts showed a significantly later onset of reinnervation and final amplitude of 28%.

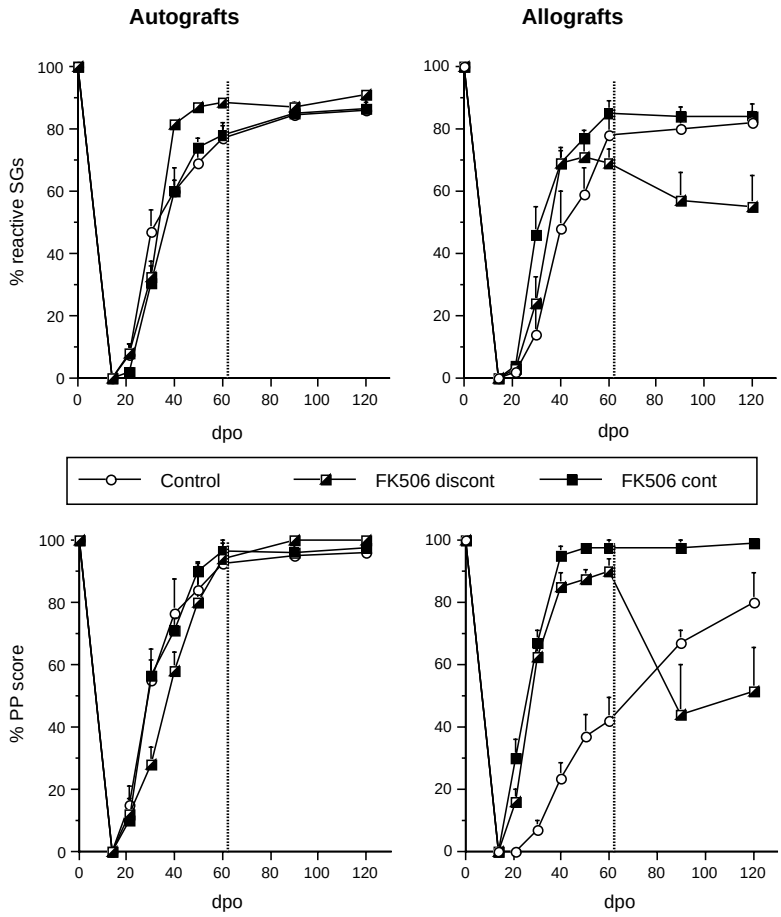

Fig. 2. Percentage of the number of reactive SGs and the PP score over time in groups of mice with sciatic nerve resection and repair with a nerve autograft (left panel) or a nerve allograft (right panel) untreated (Control) or treated with FK506. FK506 was administered during the 4-month follow-up in groups with continuous treatment (FK506 cont), and until the time indicated by vertical dotted lines in groups with discontinuous treatment (FK506 discont).

Discontinuous treatment of allografts produced the same initial course of recovery as continuous administration, but showed a marked decline after 60 dpo to a final mean CMAP amplitude of 16%. CNAPs of digital nerves were recorded earlier in those groups treated with FK506 (at 38-43 dpo as a mean; Fig. 1, Table 1) than in control autografts (45 dpo), whereas the onset in untreated allografts was significantly delayed (95 dpo). The final amplitude of CNAPs was slightly higher in autografts treated with FK506 and allografts given continuous administration than in control autografts (see Table 1). Allografts given discontinuous FK506 administration showed a decline in CNAP amplitude after withdrawal, whereas untreated allografts had a delayed recovery, resulting in final values of approximately 12%.

Sudomotor responses returned earlier (by 21-30 dpo) than motor and sensory responses in mice of all the groups, exhibiting a steep slope of reinnervation (Fig. 2). Surprisingly, in autografts, discontinuous FK506 administration elicited a slightly better recovery than the other two groups, which behaved similarly. Untreated allografts had a slightly slower course of recovery with time, but reached final levels similar to FK506-treated allografts. The allografts given discontinuous

TABLE 1. Mean onset (in days) of reinnervation, final percentage of reinnervation and functional recovery index (FRI) for each function tested, and the average for the four-months follow-up following resection and autograft (AutoG) or allograft (AlloG) repair.

	n	CMAP Plantar	CNAP Digital	SGs	PP score	Mean
Onset (day)						
AutoG	8	27.8 ± 0	45.0 ± 7.3	25.5 ± 1.7	26.8 ± 2.5	31.3 ± 2.4
AutoG + FK506 discontinuous	6	28.5 ± 1.5	38.3 ± 1.7	22.8 ± 1.5	24.8 ± 1,8	28.5 ± 0.8
AutoG + FK506 continuous	8	29.0 ± 1.0	40.0 ± 0	24.4 ± 1.6	25.6 ± 1.6	29.2 ± 0.6
AlloG	6	41.7 ± 4.8 [abc]	95.0 ± 14.3[abcde]	33.5 ± 3.2[abc]	35.0 ± 3.4 [abc]	50.6 ± 3.5 [abc]
AlloG + FK506 discontinuous	8	30.0 ± 0 [d]	43.8 ± 1,8	26.6 ± 1.6 [d]	23.3 ± 1.5 [d]	31.0 ± 0.9 [d]
AlloG + FK506 continuous	8	28.9 ± 1.1 [d]	40.0 ± 0	24.4 ± 1,6 [d]	22.1 ± 1.1[d]	28.8 ± 0.6 [d]
Final recovery (%)						
AutoG	8	55.7 ± 8.5	29.3 ± 5.3	86.0 ± 3.9	96.3 ± 3.8	66.8 ± 3.1
AutoG + FK506 discontinuous	6	55.0 ± 5.0	51.4 ± 5.4 [a]	91.4 ± 0.9	100.0 ± 0.0	74.4 ± 0.6 [a]
AutoG + FK506 continuous	8	52.7 ± 9.7	41.8 ± 5.3	86.7 ± 2.3	97.5 ± 2.5	65.6 ± 4.8
AlloG	6	28.1 ± 5.4 [abc]	12.0 ± 2.6 [abc]	82.2 ± 3.4	80.0 ± 9.3	50.6 ± 3.6 [abc]
AlloG + FK506 discontinuous	8	15.9 ± 6.9 [abc]	14.5 ± 5.9 [abc]	55.2 ± 9.7[abcd]	51.3 ±14.9[abcd]	34.2 ± 7.0[abcd]
AlloG + FK506 continuous	8	58.0 ± 6.4 [de]	37.7 ± 2.4 [de]	84.9 ± 2.9 [e]	98.8 ± 1.3 [e]	69.8 ± 2.0 [de]
FRI						
AutoG	8	2979 ± 571	1903 ± 254	7175 ± 424	8392 ± 565	5112 ± 309
AutoG + FK506 discontinuous	6	2373 ± 404	3008 ± 361	7796 ± 87	8117 ± 188	5323 ± 118
AutoG + FK506 continuous	8	2496 ± 471	2671 ± 350	7116 ± 261	8441 ± 187	4876 ± 355
AlloG	6	1292 ± 239 [a]	598 ± 191 [abc]	6634 ± 381	4657 ± 416[abc]	3295 ± 227[abc]
AlloG + FK506 discontinuous	8	1503 ± 388 [a]	1348 ± 323 [bc]	5565 ± 443	6265 ±760[abc]	3670 ± 342[abc]
AlloG + FK506 continuous	8	3207 ± 181 [de]	2523 ± 231[de]	7399 ± 302	9064 ± 205[de]	5548 ± 107[de]

$p < 0.05$ [a] vs group AutoG, [b] vs group AutoG+FK506 discontinuous, [c] vs group AutoG+FK506 continuous, [d] vs group AlloG, [e] vs group AlloG+FK506 discontinuous. Values are mean ± SEM.

FK506 administration showed a 20% loss in responsive SGs (from 72% at 50 dpo to 51% at 120 dpo).The nociceptive responses were initially obtained in proximal areas of the paw on average at approximately 22-25 dpo in autografts and in FK506-treated allografts. These PP scores showed a rapid recovery, being close to normal values by 60 dpo (Fig. 2). In allografts given discontinuous FK506 administration, the reduction in FK506 dose did not elicit the same level of recovery at 60 dpo observed with allografts given continuous administration, falling to 45% at 90 days. In untreated allografts, the mean onset was at 35 dpo, and recovery was more uniform over time (as shown by the smooth curve; see Fig. 2), achieving a final value of 80% of normal.

In summary, administration of FK506 resulted in a slightly (by about 10% as indicated by the mean of the four functions tested; see Table 1) faster onset of reinnervation in comparison with control autografts. The differences were more noticeable for functions mediated by sensory fibers (CNAP and PP) than for those dependent on efferent fibers (CMAP and SGs). The final levels of reinnervation were significantly higher in autografts treated only during the first 2 months (discontinuous administration) with FK506 than in controls. The untreated allografts demonstrated a delayed recovery and a slower rate of reinnervation, achieving significantly lower levels of recovery of CMAPs and CNAPs. Allografts given continuous FK506 administration were similar to autografts. Those given discontinuous FK506 administration showed the same course of reinnervation until 60 dpo, thereafter exhibiting a marked functional loss until 90 dpo when it was stabilized or slightly improved by 120 dpo. It should be noted that not all the mice demonstrated the same course after FK506 withdrawal; four mice displayed a marked functional decline (with abolished or only very small CNAP and CMAP amplitudes), one exhibited a reduction of approximately 50%, and the other three animals showed a mild loss or remained at levels similar to those achieved at 60 dpo.

Morphological results

All grafts were found to be in continuity with the injured nerve upon dissection at the termination of study. The suture sites showed slight thickening and marked vascularization. Quantitation of the numbers of myelinated axons confirmed that the axon density varied between the different groups (Table 2). Autografts exhibited extensive regeneration (Fig. 3A, B), with a near normal number of myelinated fibers (4801± 207 in intact mouse sciatic nerves) regenerating through the graft (Table 2). As usual in regenerated nerves, the myelinated fiber size and the myelin thickness were reduced with respect to intact nerves. However, in animals treated continuously with FK506, the mean fiber diameter was significantly higher than in the other two groups (Table 2); correspondingly, the percentage of small myelinated fibers (less than 4 μm in diameter) was lower in the group with continuous FK506 treatment (30%) than in groups untreated or receiving discontinuous FK506 (approx. 50%; Fig.

TABLE 2. Morphological analysis of regenerated nerves at the middle of the graft and at the distal nerve at four-months following resection and autograft (AutoG) or allograft (AlloG) repair of a 6-mm gap.

	Midgraft		**Distal nerve**	
Group	**Nerve area (mm^2)**	**Myelinated fibers number**	**Nerve area (mm^2)**	**Myelinated fibers number**
AutoG	0.16 ± 0.01	4470 ± 319	0.17 ± 0.03	3983 ± 335
AutoG + FK506 discontinuous	0.14 ± 0.01	4038 ± 161	0.15 ± 0.01	3130 ± 310
AutoG + FK506 continuous	0.14 ± 0.01	3949 ± 166	0.15 ± 0.01	3034 ± 298
AlloG	0.12 ± 0.02	2293 ± 424 [abc]	0.12 ± 0,02	2222 ± 381 [a]
AlloG + FK506 discontinuous	0.15 ± 0.02	1788 ± 398 [abc]	0.21 ± 0,04 [d]	2014 ± 245 [abc]
AlloG + FK506 continuous	0.17 ± 0.02	4043 ± 243 [de]	0.21 ± 0,04 [d]	3168 ± 291 [de]

$p < 0.05$ [a] vs group AutoG, [b] vs group AutoG+FK506 discontinuous, [c] vs group AutoG+FK506 continuous, [d] vs group AlloG, [e] vs group AlloG+FK506 discontinuous. Values are mean ± SEM.

4), In the distal nerve, the number of myelinated fibers was slightly lower (by 10 - 20%) than in the mid-graft, being greater, albeit not significantly, in the control autograft than in the FK506-treated groups.

In control allografts, the overall architecture of the nerve was preserved, regenerating axons being organized into small fascicles in the endoneurium (Fig. 3C). However, the perineurium was thickened and a number of extrafascicular regenerating fibers were present. Following discontinuous FK506 administration, the number of myelinated fibers was significantly lower compared to autografts and allografts given continuous FK506 administration (Table 2), but the size of myelinated fibers was similar to autograft groups, although there was a reduction in the percentage of large fibers (Fig. 4) when compared with autografts. In contrast, the appearance of allografts given continuous FK506 administration (Fig. 3D) was similar to that of autografts, although some nerves contained isolated infiltrates of monocytic cells. In regard to size, the diameter of myelinated fibers was slightly lower than that found in autografts (Fig. 4). Allografts given discontinuous FK506 treatment did not reach the same level of regeneration found in allografts given continuous administration, the number of myelinated fibers at mid-graft being significantly reduced. In five of the eight animals receiving discontinuous administration, the appearance of the regenerated nerves was good (Fig. 5), although there were areas of mononuclear cell infiltration (mainly around blood vessels) and some fascicles were no longer intact, as revealed by the occasional axon present extrafascicularly. The other three mice in this group showed a low number of myelinated fibers (less than 800), abundant

demyelinated axons (Fig. 5C), and immune infiltration. Nevertheless, the diameter distribution of the regenerated myelinated fibers was similar to the other allograft groups (Fig. 4). In contrast to all other groups, there were relatively more myelinated fibers present in the distal nerve compared to the mid-graft (2014 vs. 1788, respectively, see Table 2), probably due to preservation of myelination in the distal nerve despite demyelination of axons within the graft following FK506 withdrawal.

DISCUSSION

The present study shows that FK506 administration slightly improves the onset of target reinnervation and functional recovery following nerve transection and repair by autografts. The sustained administration in these cases does not show functional benefits in comparison with temporary treatment; in fact, the group with discontinuous administration had better recovery of digital CNAPs than the group with continuous administration.

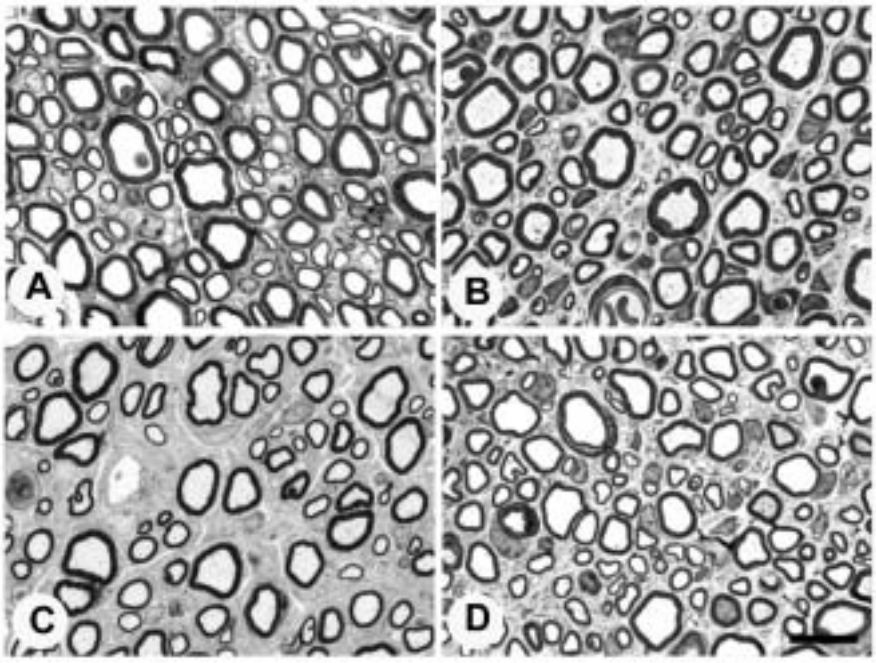

Fig. 3. Transverse semithin sections of regenerated nerves at the mid-graft four months after sciatic nerve resection and graft repair. (A) Untreated autograft; (B) autograft with continuous FK506 administration; (C) untreated allograft; (D) allograft with continuous FK506 administration. Bar = 10 µm.

Nevertheless, the morphometric findings indicate that the longer the treatment, the more marked the effect of FK506 in promoting maturity of the regenerated myelinated fibers. Moreover, continuous FK506 administration elicited the same level of recovery in allografts as found in autografts. Thus, the most important new finding is that continuous FK506 administration is necessary to achieve optimal regeneration and functional recovery in allografts. Withdrawal of

FK506 once the grafts are well tolerated by the host (i.e., at mid-point in the present study) leads to a marked reduction in function with the final outcome being worse than that found for untreated allografts.

The dose of FK506 selected for this study (5 mg/kg/day) was selected because it has been previously shown by our laboratories to accelerate maximally the rate of regeneration after crush to the sciatic nerve in the rat [52] and the mouse,[50] and to increase also the degree of target reinnervation.[49] In terms of immunosuppression, we recently found that FK506 effectively prevents rejection of nerve allograft in the mouse at a daily dose of 5 mg/kg, but is less effective at 2 mg/kg [51]; a lower, nonimmunosuppressant dose (0.2 mg/kg) does, however, accelerate the rate of regeneration.[50] For the CNAP recorded at the digital nerves, the most sensitive measurement to detect differences in our model,[38,39] administration of FK506 at the 5 mg/kg dose shortened the onset of recovery by 13% after a crush [49] and by about 12% after autograft (present study). Furthermore, the percent recovery of CNAP amplitude was increased by 35% after a crush and by 41% after autograft. Taken together, these findings indicate that FK506 elicits a similar response following either nerve crush or nerve transection with autograft repair. groups.

FK506 in autograft repair

As we previously reported using this experimental model,[38] discontinuous FK506 administration following autograft repair slightly shortens the time to onset of reinnervation and the degree of functional recovery. This effect is directly dependent on axonal regeneration since nociception and CNAP of digital nerves showed better progression than did recovery of CMAP and SGs, which require reestablishment of new synaptic contacts with their targets. In other studies involving administration of FK506 in a crush model [14,26] or following transection and autograft or isograft repair, [6,9] there was no difference in the final outcome compared to untreated groups. Consistent with these previous findings, the present study shows that the number of regenerated myelinated fibers is similar in treated and untreated groups at 4 months post-injury, although myelinated fibers are more mature in FK506-treated mice, a finding consistent with an enhanced rate of regeneration. [38]

In general, results of studies using FK506 in models normally exhibiting proficient nerve regeneration (i.e., nerve transection repaired by autograft) indicate that high immunosuppressive dosage for extended periods of time may not be justified. In this context, a surprising finding of the present study is that discontinuous FK506 administration for 2 months resulted in slightly better final outcome than did continuous treatment over 4 months, with no detrimenta

effects observed following drug withdrawal. Since FK506 has neurotrophic activity at nonimmunosuppressant doses,[16,50] low-dose administration for a short time should be considered for speeding regeneration. Alternatively, nonimmunosuppressant derivatives of FK506 demonstrating

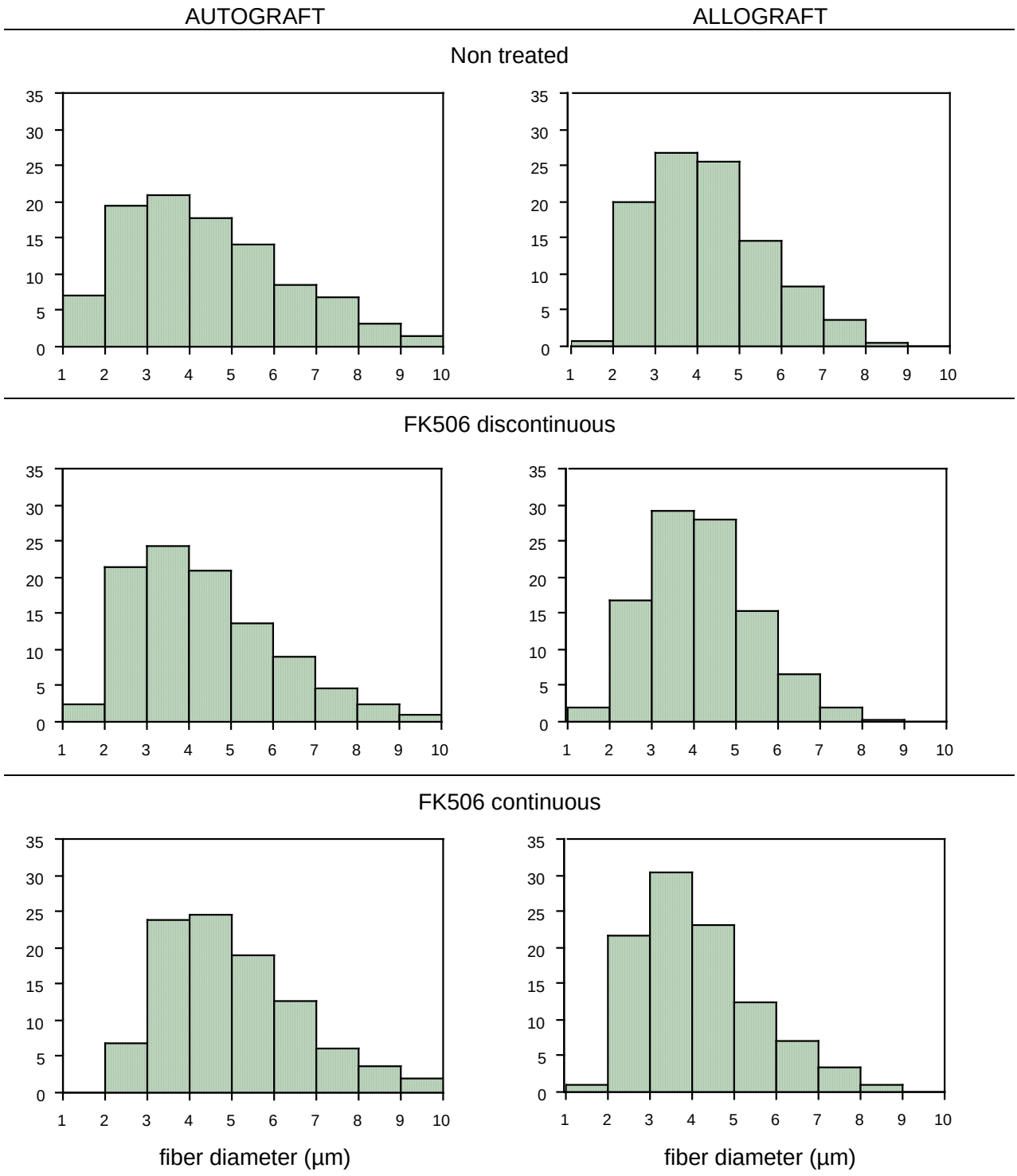

Fig. 4. Histograms showing the frequency distribution (in percentage) of myelinated fiber diameter in the regenerated nerve at the mid-graft level in groups repaired with autografts (left panels) and with allografts (right panels)

neurotrophic activity [15,45] need to be studied in these models. Although we have not explored chronic injury models in this study, the ability of FK506 to increase nerve regeneration following chronic axotomy [48] may be advantageous for improving functional recovery and reducing the consequences of target organ denervation following autograft repair of long nerves after extended periods of time.

FK506 in allograft repair

Following nerve allograft transplantation, an immunological response ensues mainly against the donor cells and myelin sheaths since their membranes carry the antigens of the major histocompatibility complex.[2,25] Rejection eliminates allogenic Schwann cells during the first weeks after grafting,[24,34,42] but does not destroy the architecture of the graft. Loss of Schwann cells renders that graft acellular, providing a poor, albeit permissive, environment for nerve regeneration.[17,20,25] Consequently, axonal regeneration is impeded or limited during the first few weeks following grafting.[16,41,42,51] At longer times, the allograft may be repopulated by recipient-derived Schwann cells migrating from both proximal and distal stumps,[22,24,34] thereby providing support and guidance to axons entering from the proximal stump.[19] Thus, as expected, regeneration and target reinnervation were significantly delayed in our untreated allograft group. Nevertheless, a nonimmunosuppressed allograft may sustain efficient regeneration only if its length is short enough to permit recipient Schwann cell repopulation, given their limited ability for migration.[31,46] Although in our model untreated allografts reached acceptable final levels of recovery, this situation is not comparable to human patients that require much longer allografts.

In contrast, when FK506 was administered at 5 mg/kg, a dose providing effective immunosuppression in the mouse,[51] the allografts were not rejected and axonal regeneration followed a course similar to that seen in autografts. We used as allograft the nerve of another animal of the same strain but different litter, since we previously showed that in this model, allografts suffer marked immunological rejection response during the first weeks after grafting. [51] Upon immunosuppression, donor Schwann cells persist in the allograft, where they are associated with and myelinate regenerating axons,[1,34] preventing migration of host cells into the graft.[34] With continuous FK506 treatment, final functional outcome and nerve morphology are equivalent to levels found in untreated autografts. The morphometric results demonstrate a slightly less mature profile of the regenerated myelinated fibers, with smaller caliber than in the autograft treated continuously with the same dose of FK506. However, the differences in size and axon/myelin ratio were not marked at the long time-point (4 months) when most regenerated fibers could have reached targets. In this context, the most important parameter in regard to functional outcome was the total number of regenerated fibers.

We found no evidence for neurotoxicity at the relatively high (5 mg/kg) dose used in our study, as found for cyclosporin A.[3] Functional outcome was considerably better in our study than in previous reports using allografts treated with lower doses of FK506. [5,40] Although not addressed in the present study, FK506 administration has also been shown to rescue peripheral nerve allografts undergoing acute rejection.[10]

After cessation of immunosuppression, donor Schwann cells are also rejected, resulting in demyelination of regenerated axons and secondary axonal degeneration. However, a proportion of donor Schwann cells survive and may persist long after the rejection response has subsided, [24] indicating that associated regenerated axons can maintain their integrity and conduct electrical impulses. Recipient Schwann cells are able to migrate into the allograft from both proximal and distal stumps, replacing rejected donor Schwann cells, and supporting the new regrowth and myelination of surviving axons. A number of investigators have examined nerve allografts following discontinuous[5,21,30,33,35,40] to complete allograft rejection with permanent functional loss. [3,53,55,56]

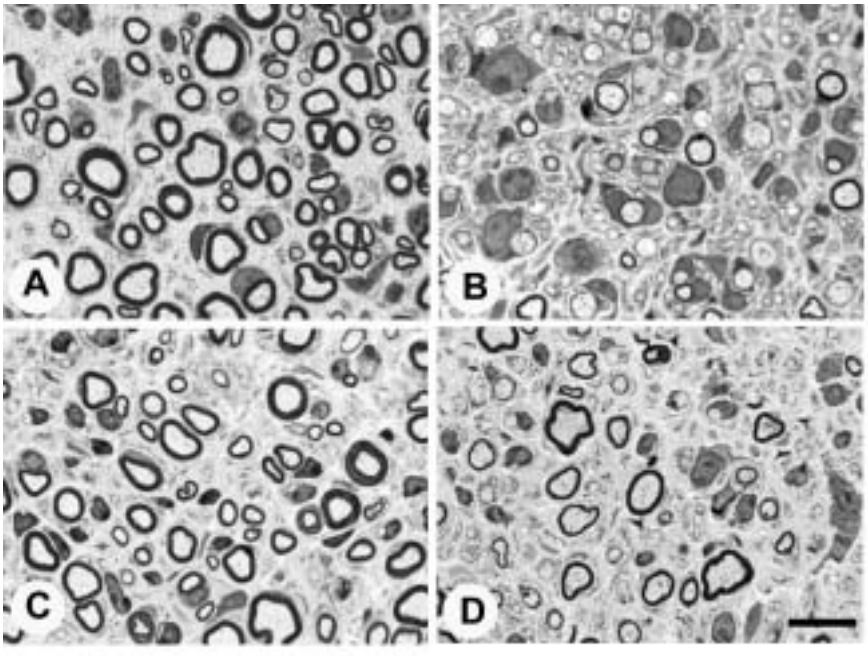

Fig. 5. Representative cross-sections of regenerated nerves four months following sciatic nerve resection and allograft repair with discontinuous administration of FK506. A & C: A mouse that had maintained functional recovery shown at mid-graft (A) and at the distal nerve (C). The appearence is similar to samples shown in Fig. 3. B & D: A mouse with evidence of rejection following withdrawal of FK506 shown at mid-graft (B) and at the distal nerve (D). Note the abundant presence of demyelinated axons at mid-graft. Bar = 10 μm.

The differences may be attributable to the immunosuppressive withdrawal regime, the intensity of rejection reflecting immunological disparity, the time and methods of study, and the length of the graft.

In the present study, we observed functional and morphological loss in the regenerated nerve following withdrawal of FK506 using allografts from another mouse, albeit the same outbreed strain. We also found different responses for different subclasses of neurons. Following FK506 withdrawal, electrophysiological evaluation of large myelinated fibers (CMAPs and CNAPs) showed a loss of approximately 50% of the recovery achieved before withdrawal, whereas sudomotor function mediated by unmyelinated sympathetic fibers showed a decline of only 20%, suggesting that myelinated fibers are more vulnerable to rejection than unmyelinated fibers. Since the immune reaction leads to sequential rejection of the donor cells in the nerve allograft,[54] but the regenerated axons are of host origin, how does one account for this preferential loss of function for myelinated fibers? This can be explained by the presence of large demyelinated axons in the rejected grafts following withdrawal of the drug (Fig. 5). The loss of host regenerated axons is related not only to the primary adverse immune effects on allogenic Schwann cells, but also to ischemia secondary to rejection of allogenic endothelial cells and the lack of protective permeability barriers in the rejected graft.[4,54]

The detrimental consequences of immunosuppression withdrawal developed despite the high levels of target-organ reinnervation that are achieved by 2 months, as described in the rat.[36] During the last month of study, however, the loss was stabilized and sensory functions (CNAPs and PP, see Figs. 1 and 2) tended to recover slightly. Longer studies are needed to determine whether recovery would reach the same level found with continuous immunosuppression. In this context, Okajima et al. [40] reported that 4 weeks following termination of FK506 administration the number of myelinated fibers in allografts decreased to about 50%. However, the grafts were not totally rejected and the number of regenerating axons recovered at 12 weeks to the level found before cessation, although remaining lower than that found in autografts. Long-term treatment with high doses of FK506 could be detrimental in nerve regeneration due to its secondary toxic effects. Our results in autografts suggest that continuous administration of FK506 for 4 months could hamper sustained regeneration. In addition, the need of chronic immunosuppressive doses of FK506 to avoid rejection and to maintain functional recovery is a serious inconvenience to the use of allografts. Pretreatment of allografts to reduce its immunogenicity and thus decrease the dose of immunosuppression needed [16, 47] would not be an ideal alternative, because pretreated allografts have a reduced number of vital Schwann cells and decreased capacity to sustain nerve regeneration. Further long-term studies on slow step-wise reduction of FK506 dosages are needed to clarify when immunosuppression may be withdrawn without risk for delayed rejection.

ACKNOWLEDGMENTS

This work was supported by grants from the Acadèmia de Ciències Mèdiques de Catalunya i Balears and the Ministerio de Ciencia y Tecnología (SAF2002-04016), Spain. We thank Fujisawa Pharmaceuticals Inc. (Osaka, Japan) for its generous gift of FK506. The technical help of Jessica Jaramillo is greatly appreciated.

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LONG TERM EFFECTS OF DISCONTINUOUS ADMINISTRATION OF FK506 ON REGENERATION THROUGH ALLOGENEIC GRAFTS IN THE SCIATIC NERVE OF THE MOUSE

MATERIAL AND METHODS

Surgical procedure and treatment

The sciatic nerve was exposed as described previously (Udina et al., in press) and a 6 mm segment was resected. The gap was repaired with an allogenic nerve graft, 6 mm long.

The animals of the group ALLO-9m+FKdisc (n=6) were treated with 5 mg/kg/day of FK506 (Fujisawa

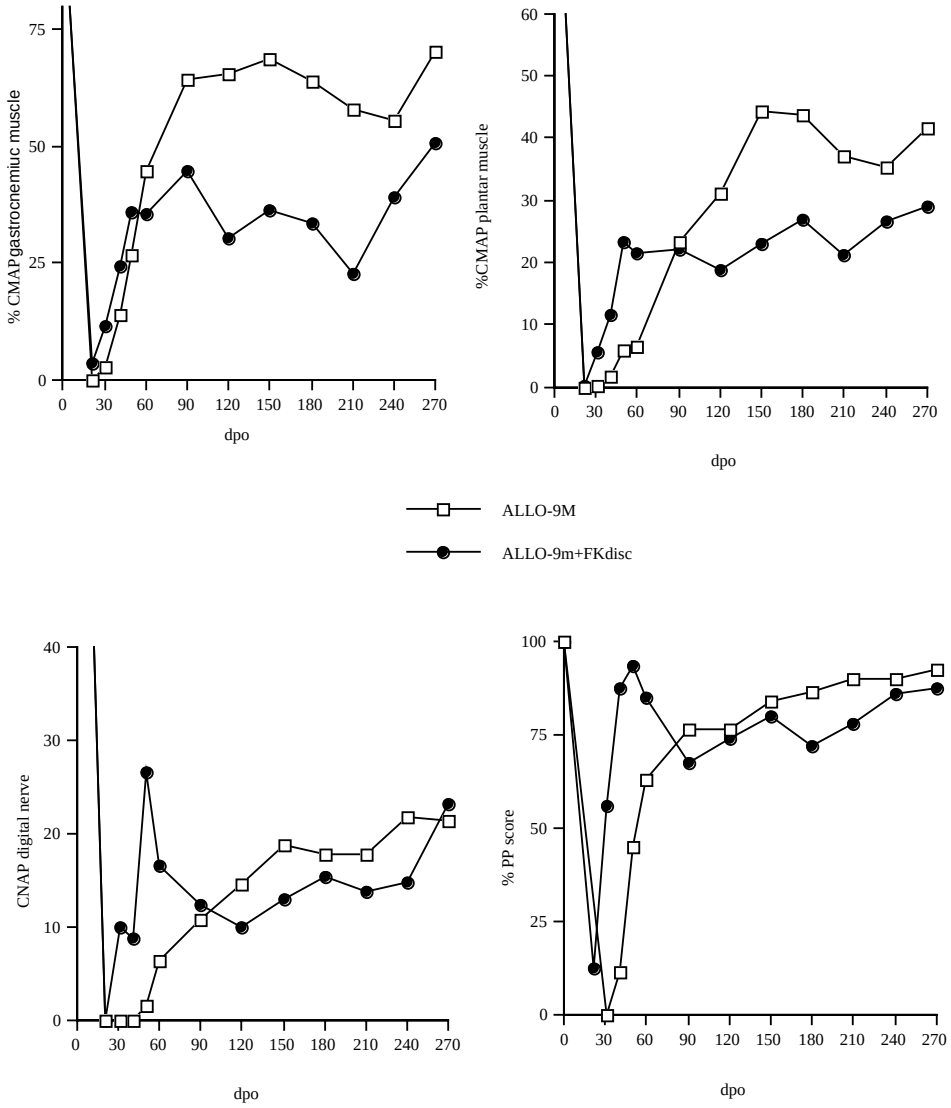

Fig 1. Percentage of the amplitude of the CMAP of gastrocnemius (a) and plantar (b) muscles, the CNAP of the digital nerve (c) and the PP score (d) over time in mice with sciatic nerve resection and allograft repair.

Pharmaceutical, Osaka, Japan) during 5 weeks and with 3 mg/kg/day for the following 4weeks, as described in the Study 5. The other group (ALLO-9m; n=6) was given saline solution for the same period of time. The animals were followed-up for 9 months.

Functional and morphological evaluation

Target organ reinnervation was periodically evaluated with a battery of functional tests described in the previous study (Study 5, Udina et al., 2003). CMAP of plantar muscle, CNAP of digital nerve and PP test were assessed at 14, 21, 30, 40, 50, 60 days, and thereafter at monthly intervals until the end of follow-up (270 days postoperation, dpo). From day 120, we also measured the conduction velocity of the regenerated nerves. The nerve was estimulated percutaneously through a pair of needle electrodes placed at the sciatic notch and then near the tibial nerve at the ankle. Compound muscle action potentials (CMAP) were recorded from the plantar muscle with monopolar needle electrodes. Compound nerve action potentials (CNAP) were recorded with needle electrodes near the digital nerves of the fourth toe. The conduction velocity of motor nerve fibers (MNCV) and of sensory fibers (SNCV) between the two stimulation points were calculated.

At 270 dpo, the regenerative nerves were dissected and processed for embedding in Epoxy resin to obtain transverse semithin sections of the mid and the distal levels of the graft, to count the number of regenerated myelinated fibers.

RESULTS

Functional results

As described in the Study 5 (Udina et al., 2003), allografts treated discontinuosly with FK506 showed the same initial course of recovery as in cases treated with continuous administration, with a significantly earlier onset of reinnervation than in untreated allografts. However, the functional recovery shown a marked decline after 60 dpo (Fig 1). There

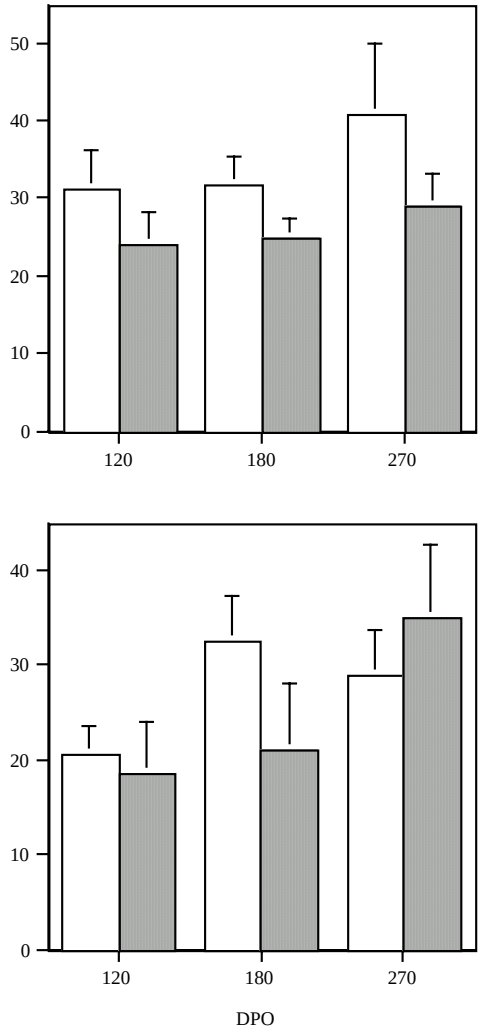

Fig. 2. Motor and sensory nerve conduction velocities of the sciatic nerve in the untreated group (white columns) and in the group that received a short course of FK506 (grey columns).

was a 30% loss of the nociciceptive recovered function, until day 90, from when it started to improve. Mean CMAP of gastrocnemius muscle showed a 30% loss in amplitude between 90 and 120 dpo, and did allografts. However, recovery showed a marked decline after 60 dpo in group ALLO-9m+FKdisc (Fig. 1). The mean CMAP of not improved until day 210, reaching final levels of only 50%, lower than in the untreated group. In contrast, there was not a marked loss of the mean plantar CMAP amplitude after withdrawal of FK506, although there has wide variability between mice. Two animals had completely abolished responses at days 90 and 120 respectively; in these animals polyphasic potentials of low amplitude and long latency were recorded one month later, and increased in amplitude with time. Another mouse showed an earlier decline at 60 day and fast recovery (to 50% of amplitude at 150 dpo), whereas another had only a slight loss of motor function at day 150 recovered one month later. Nevertheless, the treated group did not reach a level of recovery as good as in the untreated group (30% vs 40%). The MNCV of group ALLO-9m+FKdisc was slightly slower during the last 5 months of follow-up in comparison with group ALLO-9m (Fig. 2).

The loss of sensory function was comparatively more marked. There was a 30% loss of the recovered nociceptive function until day 90, from when it improved again to achieve similar final values than in group ALLO-9m. On another hand, the digital CNAP amplitude declined about 60% between 50 and 120 days, and recovered later than nociceptive responses. The same animals that had abolished plantar CMAPs, did have complete loss of the digital CNAPs at 90dpo, which reappeared at 120-210 dpo. Despite this marked loss, the late recovery was good and at the end of follow-up, the mean CNAP amplitude (23%) was similar to that of the untretaed group. There were no significant differences for the SNCV between the two

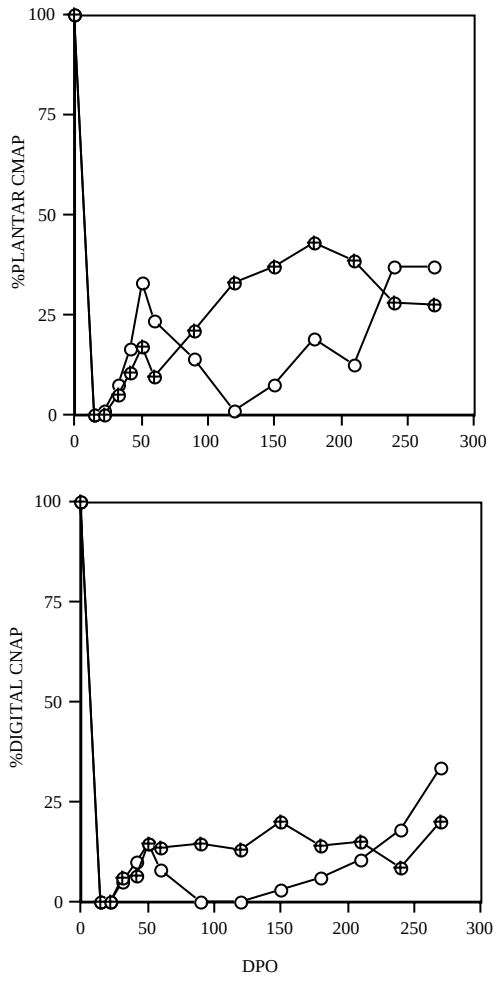

Fig 3. Evolution of plantar CMAP and digital CNAP amplitude of four animals in the group discontinuously treated with FK506. Two animals had abolished responses (crossed circles), whereas the other two showed a slight loss and a fast recovery (white circles), but the final recovery was similar for all of them.

compared groups, despite it was slightly faster in group ALLO-9m+FKdisc than in the untreated group at 9 months (Fig. 2).

When studying the behavior of the animals with discontinuous FK506 treatment individually, two animals presented a marked loss of the recovered function after withdrawal of the drug (Fig. 3, crossed dots), whereas three mice showed a slight earlier loss and recovered faster to a stable level (Fig. 3, white dots). Another two had an intermediate behavior (not shown). Nevertheless, the evolution after withdrawal did not determine the final outcome reached, since by 9 months the levels of functional recovery were similar for all the animals of this group.

Morphological results

All grafts were found to be in continuity with the injured nerve upon dissection at 270 dpo. The general microarchitecture was preserved in all the nerves. The number of myelinated fibers (Table 1) was significantly lower in the treated animals (1863±234 at midgraft and 1451±125 distally) than in the untreated group (3014±257 and 2300±179 respectively). In one of the animals that received FK506 during the first two month, the graft appeared swollen and there was an important amount of immunitary cells, and some demyelinated axons, although the number of regenerative fibers was 1500. The grafts in the other treated mice showed a similar aspect to that observed in untreated allografts

.

TABLE 1. Morphological analysis of regenerated nerves at the mid-point of the graft and distally after 9-months of the interposition of an allograft to repair a 6-mm gap resection.

		Nerve area (mm^2)		**N myelinated fibers**	
	Nerve cable	**Medial**	**Distal**	**Medial**	**Distal**
ALLO-9M	4/4	0.017±0.02	0.12±0.013	3014±257	2300±179
ALLO-9M+FK disc	4/4	0.017±0.04	0.17±0.04	1863±234[a]	1451±125[a]

$p < 0.05$ [a] vs group ALLO-9M

DISCUSSION

As discussed in the previous study, withdrawal of immunosuppression leads to an important loss of the function reached by the allografts. Although unmyelinated fibers are able to recover function in one month and myelinated fibers in two months, recovery is slow and incomplete, and does not reach the levels found in untreated animals, despite that repair with fresh allografts (as shown in this study) allows only for a limited regeneration in comparison to autografts or allografts treated continuously with FK506.

Sensory responses conveyed by large (CNAPs) and small (nociception) fibers achieved final levels that were equal to those found in the

untreated group 9 months after lesion. In contrast, motor functions remained at lower levels. There are some clinical reports that describe a maintained sensory recovery in patients receiving long nerve allografts, despite withdrawal of immunosuppression (Bain, 1998), probably due to the higher sensitivity of motor axons to immune rejection in comparision to sensory axons (Midha et al., 1997).

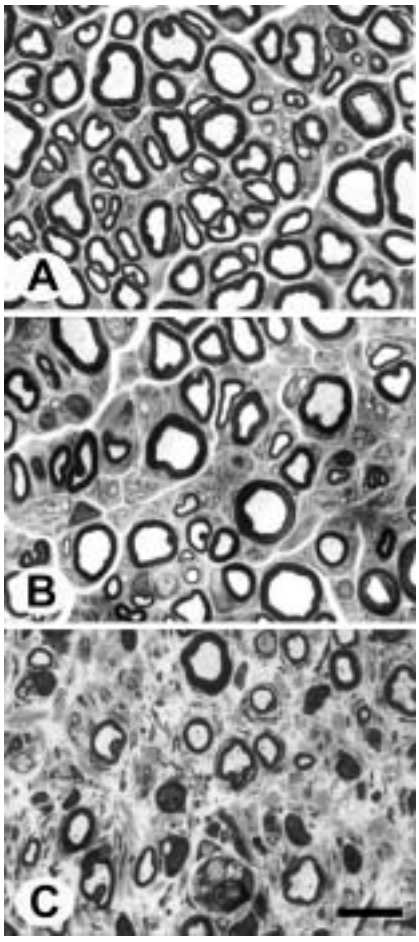

Fig 4. Cross-sections of regenerated nerves at mid-graft 9 months following sciatic nerve resection and allograft repair in a representative animal of group ALLO-9M (A) and of group ALLO-9M+FK disc (B). Detail of the graft with infiltrated immune cells in the unique animal of group ALLO-9M+FK that still had evidence of rejection (C). Bar =10μm.

Moreover, the inability of axons to recover the levels that they reached before withdrawal of FK506 can be due to the chronicity of the situation. It is known that prolonged denervation of the distal nerve stump determines a poor functional recovery due to disruption of the basal laminae (Giannini and Dick, 1990), progressive fibrosis and collagenization (Weinberg and Spencer, 1978; Vuorinen et al., 1995) and atrophy of Schwann cells (Pellegrino and Spencer, 1985). It seems that a time window (the first month) exists during which Schwann cells in the distal nerve stump stimulate and support axonal growth. Two months after delayed nerve regeneration, motor reinnervation is reduced (Sulaiman et al., 2002). Although immunosuppressed allografts allowed reinnervation before withdrawing the treatment, the immune rejection response led to marked loss of donnor Schwann cells and ischemia secondary to rejection of endothelial cells (Zalewski et al., 1995). Schwann cells from the host stumps may migrate to repopulate the graft (Midha et al., 1994), but these cells will not be so reactive as just after acute nerve injury. Thus, the graft itself but also the distal nerve will represent a poor environment for re-regeneration of the axons. Therefore, allografts rejected after discontinuation of immunosuppressive treatment will become a similarly poor substrate for regeneration that untreated fresh allografts, but with the disadvantage of the reduced reactive response in the host nerve by two months of delay from the injury.

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Efectes de l'FK506 sobre la regeneració després de reseccions nervioses severes reparades mitjançant pròtesis cèl.lulars

FK506 enhances regeneration of axons across long peripheral nerve gaps repaired with collagen guides seeded with allogeneic Schwann cells.
Udina E, Rodríguez FJ, Verdú E, Espejo M, Gold BG, Navarro X.
(Manuscrit, acceptat per publicar a Glia)

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FK506 ENHANCES REGENERATION OF AXONS ACROSS LONG PERIPHERAL NERVE GAPS REPAIRED WITH COLLAGEN GUIDES SEEDED WITH ALLOGENEIC SCHWANN CELLS.

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ABSTRACT

We assessed the effects of FK506 administration on regeneration after a 6-mm gap repair with a collagen guide seeded with allogeneic Schwann cells (SCs) in the mouse sciatic nerve. SCs were isolated from predegenerated adult sciatic nerves and expanded in culture using a defined medium, before being seeded in the collagen guide embedded in Matrigel. Functional reinnervation was evaluated by noninvasive methods to determine recovery of motor, sensory and autonomic functions in the hindpaw over 4-months postoperation. Histological analysis of the regenerated nerves was performed at the end of the study. Using simple collagen guides for tubulization repair, treatment with an immunosuppressant dose of FK506 (5 mg/kg/day) resulted in significant improvement of the onset and the degree of reinnervation. While the introduction of allogeneic SCs did not improve regeneration versus a collagen guide filled only with Matrigel, treatment with FK506 allowed for successful regeneration in all the mice, and significant improvement in the levels of functional recovery. Compared to the untreated group, there was greater survival of transplanted prelabeled SCs in the FK506-treated animals. Morphologically, the best nerve regeneration (in terms of nerve caliber and numbers of myelinated axons) was obtained with SC-seeded guides from FK506-treated animals. Thus, FK506 should be considered as an adjunct therapy for various types of tubulization repair.
***Key words**: cellular prosthesis, FK506, reinnervation, Schwann cell, tube repair.*

INTRODUCTION

Following severe nerve injuries with loss of nerve tissue, the proximal and distal stumps need to be reconnected to allow regenerating axons to find the distal pathway and eventually reinnervate their target organs. For surgical repair, an autologous graft obtained from another nerve of lesser functional importance is usually employed to bridge the gap (Millesi, 1981; Lundborg, 2000). However, autograft repair implies some inconveniences such as the sacrifice of a healthy nerve from the patient, the need of a second surgical step, the limited supply of donor nerves, and the mismatch between nerve and graft dimensions. These problems are more marked with longer gaps. Allografts may provide an alternative to autografts, but their high antigenicity forces systemic immunosuppression to avoid immune rejection (Evans et al., 1994).

A promising alternative to the use of grafts is the interposition of an artificial nerve guide. Nevertheless, tubulization with simple guides usually fails when bridging relatively

long gaps of 6-mm in the mouse (Butí et al., 1996; Gómez et al., 1996), 15-mm in the rat (Lundborg et al 1982) and 30-mm in primates (Mackinnon and Dellon, 1990; Krarup et al., 2002). Due to this limited capacity of artificial guides to support regeneration across long gaps, the introduction of factors that promote axonal regeneration into the guides has been assayed extensively. Considering the importance of the Schwann cells (SCs) in creating a favorable environment for nerve regeneration, the construction of cellular prostheses consisting of a nerve guide seeded with isolated SCs has been attempted. The introduction of SCs into the guide prior to implantation mimics the composition of a nerve graft and provides regenerating axons with an adequate supportive environment. Several studies have shown that such cellular prostheses significantly improve nerve regeneration compared to simple tubes (i.e., those not seeded with exogenous cells) (Guénard et al., 1992; Keeley et al., 1993; Kim et al., 1994; Ansselin et al., 1997; Levi et al., 1994; 1997; Rodríguez et al., 2000). However, immune compatibility between donor cells and host is an important factor that affects the success of cellular prostheses (Guénard et al., 1992; Rodríguez et al., 2000). We previously reported (Rodríguez et al., 2000) that transplants of autologous SCs resulted in similar levels of recovery compared to autografts, but higher functional recovery and numbers of regenerated fibers reaching the distal nerve than transplants of isologous and allogeneic SCs. In contrast, allogeneic SCs did not improve regeneration with respect to acellular guides (Rodríguez et al., 2000).

An additional approach for improving the regenerative capacity of nerve guides is co-adjuvant treatment with pharmacological agents that can enhance the rate of nerve regeneration and the degree of functional recovery. FK506 is an immunophilin ligand with strong immunosuppressive effects that also has potent neurotrophic and neuroprotective actions (for reviews see Gold, 2000; Guo et al., 2001; Gold and Villafranca, 2003; Gold et al, 2004). We have shown that FK506 increases the rate of axonal regeneration and also increases the level of target reinnervation after peripheral nerve injuries (Gold et al., 1995; Udina et al., 2002; Udina et al 2003a). Administration of FK506 would be of interest in cases where this double effect, immunosuppression and neurotrophism are required. In this respect, administration of FK506 for immunosuppression has also been found to speed functional recovery in hand transplantation patients (Dubernard et al., 1999; Jones et al., 2000; Margreiter et al., 2002) and allowed regeneration in severe nerve lesions that, due the limitation of autologous nerve grafts, needed allogeneic material (Bain, 2000). In the present study we examined the effects of FK506 on nerve regeneration through collagen nerve guides alone or seeded with transplanted allogeneic SCs when used to repair a long gap in the mouse sciatic nerve.

MATERIAL AND METHODS

Surgical procedure and experimental groups

Operations were performed under pentobarbital anesthesia (60 mg/kg i.p.) on five groups of female OF1 mice, aged 2.5 months. All experimental procedures were approved by

the Ethics Committee at our institution. First, the saphenous nerve was cut in the femoral space and a long segment of the distal stump removed to prevent its regeneration to avoid reinnervation by collateral sprouting (Udina et al., 2003a). The right sciatic nerve was then exposed at the midthigh, transected at 45 mm from the tip of the third digit and a 6-mm distal segment resected. Collagen guides (Integra LifeSciences, Plainsboro, NJ) of 1 mm i.d., 0.5 mm wall thickness and 8 mm in length were implanted with one 10-0 suture into each stump of the nerve leaving a final interstump gap of 6 mm. The wound was sutured and disinfected with povidone-iodine. In order to avoid autotomy after denervation, animals were treated with amitriptyline (Navarro et al 1994).

According to the implant and the treatment, the experimental groups evaluated were:

- Group C (n=9): collagen guide filled with saline solution;
- Group C+FK506 (n=9): same as C treated with FK506;
- Group M (n=9): collagen guide filled with Matrigel (Collaborative Research Inc., Bedford, MA) at 4 mg/ml;
- Group SC (n=7): collagen guide filled with Matrigel seeded with 200,000 allogeneic SCs;
- Group SC+FK506 (n=9): same as SC treated with FK506.

Mice in groups treated with FK506 were given daily subcutaneous injections of 5 mg/kg of FK506 (Fujisawa Pharmaceuticals, Inc., Osaka, Japan) diluted in saline solution, beginning on the day of operation and until 100 days postoperation (dpo). The dose was reduced to 3 mg/kg for 20 more days. Mice of the other groups received an equivalent volume of saline solution. In order to prevent possible infections, all the mice were treated with 100 mg/kg s.c. of amoxicillin (Clamoxyl, Glaxo Smith Kline) during the first 4 days.

Schwann cell cultures

SCs from female OF1 mice, aged 2 months, were isolated following a previously reported method (Verdú et al., 2000). The animals were anesthetized (60 mg/kg pentobarbital i.p.), the sciatic nerve was exposed and cut at the upper thigh, and the wound was sutured and disinfected. Seven days later, animals were reanesthetized to remove the in vivo degenerating nerves under asepsis. The nerve segments were stored in Hanks's balanced solution (HBSS, Sigma, St Louis, MO) with Ca^{2+} and Mg^{2+} and the medium was kept cold in a refrigerated dish. The epineurium and connective tissue were stripped off and the nerves were mechanically and enzymatically (0.25% trypsin, 0.1% collagenase A, 0.1% DNAse-I in 1 ml HBSS at 37ºC for 2-3 hours) dissociated. Addition of Dulbecco's minimum essential medium F-12 Ham (DMEM/F-12, Sigma) inactivated the enzymes and the cell mixture was recovered by centrifugation (900 rpm, 7 min). The cell suspension was seeded onto microplates pre-coated with poli-L-lysine (10 µg/ml) and incubated with a defined culture medium under 5% CO_2 at 37ºC. Seven days later, cells from confluent SC cultures with a 75% of purity were detached with trypsin

(0.05% w/v)-EDTA (0.02%) for 2-3 min and the cell mixture was recovered by centrifugation. Cells were resuspended in culture medium mixed with Matrigel at a final concentration of 4 mg/ml. Then, the volume was adjusted to obtain a final number of 200,000 cells per tube. Collagen guides presoaked in culture medium were filled with the SC suspension and kept at 37ºC for 3-4 hours.

Functional evaluation

Functional reinnervation of target organs in the hindpaw was tested at several intervals after denervation up to 120 dpo by means of a battery of noninvasive neurophysiological tests (Navarro et al 1994b; Navarro et al., 2001). Regeneration of large myelinated nerve fibers was assessed by nerve conduction tests. The sciatic nerve was stimulated percutaneously through a pair of needle electrodes placed near the sciatic notch and the compound muscle action potential (CMAP) was recorded from the gastrocnemius and plantar muscles with microneedle electrodes. For sensory nerve conduction tests, the recording electrodes were placed near the tibial nerve at the ankle and near the digital nerves of the fourth toe to record the compound sensory nerve action potential (CNAP). The evoked compound action potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S) at settings appropriate to measure the amplitude from baseline to the maximal negative peak and the latency from stimulus to the onset of the first negative deflection.

Reinnervation by small fibers was evaluated by testing sympathetic sudomotor and nociceptive responses. Sweating was stimulated by injection of pilocarpine nitrate (5 mg/kg s.c.). Ten minutes later, a silicone material (Elasticon, Kerr Co., Romulus, MI) was applied over the plantar surface of the hindpaw. As the material hardened, it retained the impressions made by the sweat droplets emerging from individual sweat glands (SGs). The number and distribution of SG impressions were determined under a dissecting microscope using transillumination. Recovery of pain sensitivity was tested by light pricking with a needle under a dissecting microscope in five areas, from the most proximal pawpad to the tip of the second digit on the plantar surface of the denervated paw. A score was assigned from no response (0), reduced or inconsistent response (1), to normal reaction (2) in each area tested in comparison with responses in the contralateral intact paw, and the 5 scores added into a paw pinprick score (PP).

For normalization of the data, values obtained after operation were expressed as percentage of preoperative values for each individual mouse and plotted against time. For each functional parameter indicative of the degree of reinnervation (CMAP and CNAP amplitudes, SG number and PP score), we determined the day of the first response after denervation (when there was no reinnervation an arbitrary value of 150 days was assigned) and the percentage of maximal recovery achieved during the follow-up. We also calculated an overall functional recovery index

(FRI) representing the area under the reinnervation curve.

Morphological evaluation

At the end of the study, animals were reanesthetized, the operated nerve dissected from surrounding tissues, and a segment including the guide and 1 cm distal nerve was harvested. The nerves were fixed in glutaraldehyde-paraformaldehyde (3% - 3%) in 0.1 M cacodylate buffer (pH 7.4, 4h, 4ºC), postfixed in OsO_4 (2%, 2h), and dehydrated through ethanol series. After the first dehydration, the tissues were stained with uranyl acetate (2.5%, 12 h) in 70% ethanol. The samples were then processed for embedding in Epon. Transverse semithin sections (0.5 µm) of the entire nerve at midpoint and distal to the guide were made with an ultramicrotome (LKB 6802), stained with toluidine blue and examined under light microscopy. Images were acquired with an Olympus DP50 camera to computer. For estimating the total number of regenerated myelinated fibers, fibers were counted in systematically selected fields covering a 40% of the total nerve area. Counting and measurement of the transverse area of the nerve were made using the NIH-Image program. The total number of myelinated fibers in the nerve was estimated from the area occupied by the fibers in the counted fields. When there was no nerve regeneration at the cross-sectional level, a value of zero was entered for nerve area and the number of myelinated fibers.

Implant evaluation

In 8 additional mice nerve guides were filled with prelabeled SCs to determine whether the cells in fact survived transplantation and confirm their presence in the nerve guides following the regenerative process.

Cultured SCs were prelabeled with the red cell linker PKH26-GL (Sigma). SCs were centrifuged and resuspended in 250 µl of the commercial diluent C containing 1 µl of the dye and gently mixed for 2 min. The reaction was stopped by adding medium and the excess of dye was eliminated by centrifugation. Labeled SCs were seeded into collagen guides as above. Half of the mice received FK506 and the other half were untreated. After 30 days, the regenerated nerve was re-exposed and carefully pulled out from the tube, fixed in 4% paraformaldehyde for 30 minutes, and cryoprotected with 30% saccarose in phosphate buffered saline (PBS). The samples were longitudinally sectioned in a cryostat (Tissue Tek-II, 4550) at 18 µm and mounted on gelatin-coated slides. Some sections were stained against S100 protein. The sections were incubated for 1 h in 0.1 M PBS with Triton X-100 (Fluka) and 5% fetal cow serum, and then for 3 h in 0.1 M PBS with rabbit antiserum to S-100 (Incstar), washed in PBS-Triton, and finally incubated for 2h in 0.1 M PBS with donkey antirabbit cyanine-2-labeled immunoglobulin G (1:200; Jackson Immuno-research).

Statistical analysis

All data are expressed as mean ± SEM. Statistical comparisons between groups were made by ANOVA followed by a post-hoc

Fisher test. Differences were considered significant at $p<0.05$.

RESULTS

Functional results

Table 1 shows the mean values for onset and maximal percentage of reinnervation for all functions tested. Collagen guides alone showed a low capability to sustain regeneration over the 6-mm long gap; only 3 out of 9 mice of group C showed functional reinnervation (Table 1). CMAPs were recorded in these 3 mice in the gastrocnemius muscle from 60 dpo, and in the plantar muscle between 90 and 120 dpo (Fig. 1). Recordable tibial CNAPs were found from 90 dpo, whereas we did not obtain CNAPs in the digital nerves over the 4-months follow-up (Fig. 2). Sudomotor reinnervation, judged from the reappearance of SG secretion, was evident from days 60-90 in three animals, and the final mean value of recovery was only 12% (Fig. 3).

TABLE 1. Reinnervation success rate, onset day and maximal degree of reinnervation for each function tested during 4 months follow-up after resection and tube repair leaving a 6 mm gap in the five groups of mice studied.

Group	**C**	**C+FK506**	**M**	**SC**	**SC+FK506**
Reinnervation	3 / 9	6 / 9	4 / 9	5 / 7	9 / 9
Onset day					
CMAP gastroc	127 ± 13	80 ± 14[a]	120 ± 12[b]	97 ± 16	49 ± 6[acd]
CMAP plantar	137 ± 7	99 ± 13[a]	123 ± 12	110 ± 14	66 ± 8[abcd]
CNAP tibial	137 ± 7	86 ± 17[a]	121 ± 11	97 ± 15[a]	51 ± 6[abcd]
CNAP digital	150 ± 0	127 ± 11	150 ± 0	137 ± 9	100 ± 16[abcd]
PP score	121 ± 14	83 ± 17	116 ± 14	101 ± 18	48 ± 4[acd]
SGs	121 ± 14	88 ± 17	106 ± 17	93 ± 16	49 ± 7[ac]
Recovery (%)					
CMAP gastroc	10.6 ± 5.8	29.3 ± 8.4	16.4 ± 6.9	16.2 ± 7.3	44.2 ± 11.9[acd]
CMAP plantar	0.7 ± 0.6	11.2 ± 5.9[a]	4.2 ± 3.2	6.1 ± 3.6	13.1 ± 4.1[a]
CNAP tibial	9.7 ± 5.0	23.7 ± 10.4	17.1 ± 5.8	27.3 ± 10.9	42.5 ± 10.1[a]
CNAP digital	0.0 ± 0	7.0 ± 3.1[a]	0.0 ± 0[b]	5.0 ± 3.4	8.8 ± 3.4[ac]
PP score	24.4 ± 10.3	35.5 ± 12.0	27.8 ± 12.1	28.3 ± 10.8	68.7 ± 10.7[abcd]
SGs	11.6 ± 9.0	31.1 ± 10.9	29.4 ± 12.2	30.1 ± 10.5	42.1 ± 15.9[a]

Values are mean ± SEM. Groups were repaired with a collagen guide (C), filled with Matrigel alone (M), or containing allogeneic Schwann cells (SC), either untreated or treated with FK506.

$P<0.05$ vs [a] group C, [b] group C+FK506, [c] group M, [d] group SC

Recovery of nociceptive responses showed a similar progression than sudomotor function reaching a mean final PP score of 24%.

Treatment with FK506 in group C+FK506 doubled the success of regeneration (6 of 9 mice) and improved reinnervation. The first plantar CMAPs reappeared in half of the animals at 50-60 dpo, and the final mean

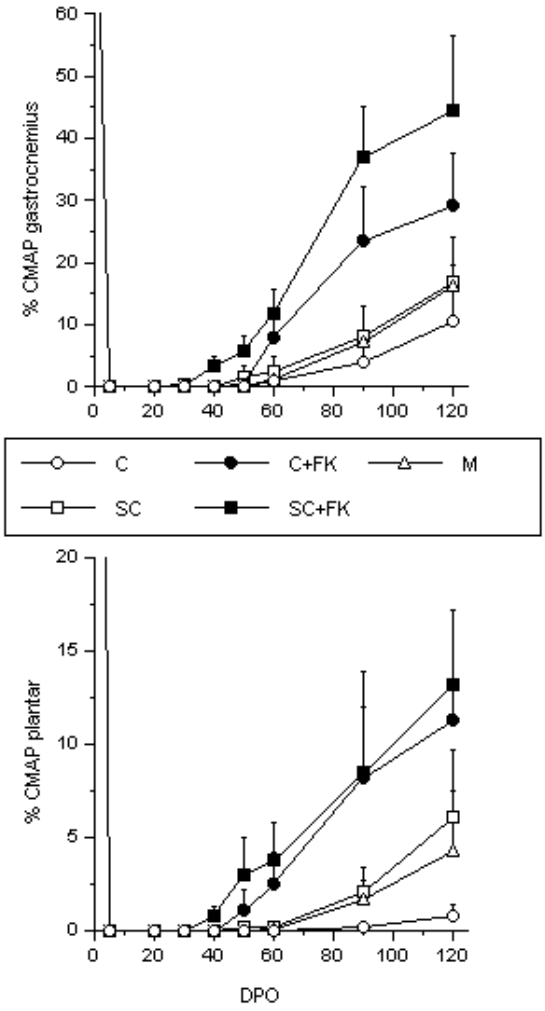

Fig 1. Percentage of the amplitude of the CMAP of gastrocnemius (top) and plantar (bottom) muscles over time in the five groups of mice studied. Preoperative values (0 day) were considered as100% (not shown in the graphs) and fell to 0% at the following test 7 days after nerve lesion. Bars are the SEM.

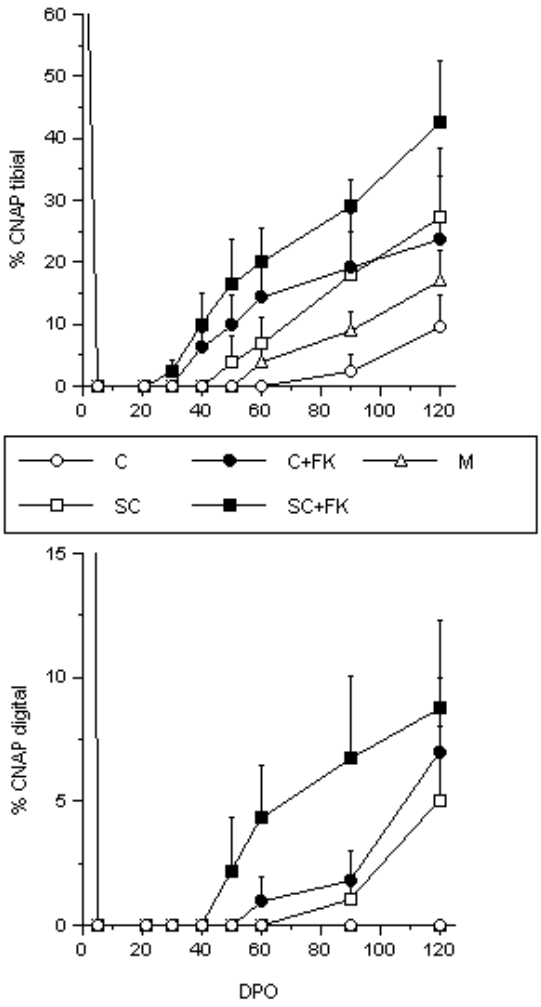

Fig. 2. Percentage of the amplitude of the CNAP of tibial (top) and digital (bottom) nerves over time in the five groups of mice studied. Preoperative values (0 day) were considered as100% (not shown in the graphs) and fell to 0% at the following test 7 days after nerve lesion. Bars are the SEM.

recovery was 11%,significantly higher than that obtained in group C (0.7%) (Table 1). CNAPs in the digital nerve were recorded in 4 mice of this group, with final mean amplitude of 7%. The percentage of recovery of sudomotor function was 31%, more than twice that reached by group C (12%) (Fig. 2). The onset of nociceptive responses was earlier and followed a steeper curve compared to group C, reaching a final PP score of 36%.

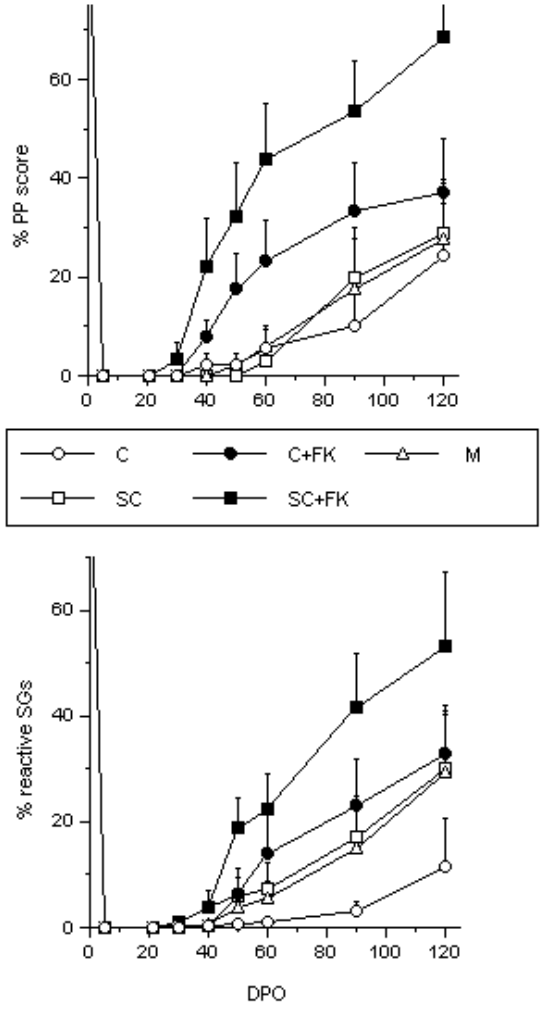

Fig. 3. Percentage of the nociceptive PP score (top) and of the number of reactive SGs (bottom) over time in the five groups of mice studied. Preoperative values (0 day) were considered as100% (not shown in the graphs) and fell to 0% at the following test 7 days after nerve lesion. Bars are the SEM.

Addition of SCs inside the collagen guide in group SC increased the proportion of successful reinnervation (to 5 of 7), whereas in group M (with Matrigel alone in the guide) only 4 of 9 mice showed effective reinnervation (Table 1). However, muscle reinnervation, as shown by the evolution of CMAPs in gastrocnemius and plantar muscles, was similar in groups SC and M, being higher for both than in group C but lower than in group C+FK506 (Fig. 1). On the other hand, the amplitude of tibial CNAPs exhibited a better course in group SC than in group M (Fig. 2). Only 2 animals in group SC demonstrated recordable digital CNAPs, with mean final amplitude of 5%, whereas none were obtained in group M. The onset, evolution and final recovery of the number of reactive SGs and of the PP score were similar in groups SC and M (Fig. 3). Both had better results than found in group C, albeit worse than in group C+FK506.

Treatment with FK506 in animals with SCs transplanted inside the guides allowed for successful reinnervation in all the mice of group SC+FK506 (9 of 9). The onset of reinnervation of most types of nerve fibers tested occurred significantly earlier than in the other groups (Table 1). The digital CNAPs were first recorded at 50-60 dpo in half of the mice, and by the end of follow-up only three had not recordable responses. The mean final values of reinnervation were also higher in group SC+FK506 compared to the other groups, being statistically significant comparedto group C for all the tests, and versus all other groups for nociceptive function and gastrocnemius muscle reinnervation.

The integrated functional recovery index, which takes into account the temporal evolution of reinnervation in targets located at the hindpaw (i.e. the mean of the indexes for plantar CMAP, digital CNAP, PP score and number of reactive SGs), is shown in Fig. 4. Group SC+FK506 showed the highest values, followed by groups C+FK506 and SC.

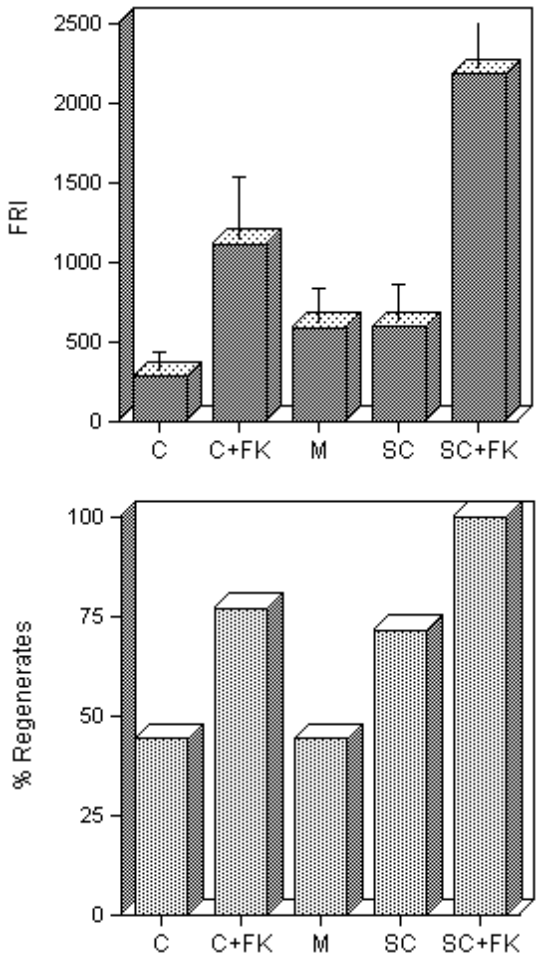

Fig. 4. Histograms of the mean functional recovery index (FRI) of targets located at the distal hindpaw (plantar muscle, digital nerve, nociception and sweating), and of the percentage of regenerates (under histological corroboration) in the five groups of mice.

Morphological results

Upon dissection, we found that the collagen guides were partially degraded, to variable degree between individual animals. Light microscopy at the midpoint of the guide demonstrated a regenerated nerve in those animals exhibiting functional reinnervation, although its caliber was smaller than the normal sciatic nerve. In groups C and C+FK506, one animal in each group had a small regenerating nerve inside the tube, despite absence of functional reinnervation of the distal targets (Fig. 4).

The nerves inside the collagen guides were all highly vascularized, but there was some variability in their microscopical architecture. In some, a significant amount of fibroblasts and connective tissue surrounded small fascicles of myelinated and unmyelinated regenerative fibers. Others showed more densely packed fascicles with a higher density of regenerated fibers (Fig. 5). In non-seeded guides, group C+FK506 had a mean of approximately 800 myelinated fibers at mid-guide and in the distal nerve, being double that in group M and four-times more that in group C (Table 2). The groups with guides seeded with SCs had approximately 960 myelinated fibers at the mid-guide, whereas distally the number was slightly higher in group SC+FK506 (920) than in group SC (600). In contrast to the immunosuppressed SC guides, one of the animals in group SC showed some areas with demyelinated fibers at mid-tube, and another exhibited moderate inflammatory infiltration.

TABLE 2. Morphological analysis of regenerated nerves at mid tube and at the distal stump 4 months after resection and tubulization repair of a 6-mm gap.

Group	Regenerate	Mid-guide		Distal nerve	
		Nerve area (mm^2)	No myelinated fibers	Nerve area (mm^2)	No myelinated fibers
C	4 / 9	0.071 ± 0.037	198 ± 130	0.031 ± 0.0.01	185 ± 111
C+FK506	7 / 9	0.088 ± 0.015	800 ± 253	0.078 ± 0.022	793 ± 333 [a]
M	4 / 9	0.050 ± 0.023	419 ± 174	0.041 ± 0.026	376 ± 189
SC	5 / 7	0.132 ± 0.031	966 ± 423	0.072 ± 0.020	599 ± 260
SC+FK506	9 / 9	0.106 ± 0.018 [a]	962 ± 257 [a]	0.100 ± 0.014 [ac]	920 ± 297 [ac]

Values are mean ± SEM. $P < 0.05$ vs [a] group C, [b] group C+FK506, [c] group M.

Implant evaluation

One month after transplantation, a regenerative nerve cable was found in the 4 FK506-treated mice and in only 2 untreated mice. Transplanted PKH26-labeled SCs were visualized in the regenerated nerves by the red fluorescence of the label. There was a clear row of fluorescent cells through the longitudinal section of the cable, whereas non-labeled cells were seen in the proximal and distal stumps, indicating that the transplanted SCs survived in the guide and did not migrate. The density of fluorescent cells was higher in treated than in untreated animals (Fig. 6 A and B). When the samples were permeabilized and stained for S-100, rows of S-100 immunoreactive cells were found in the same location occupied by PKH26-labeled cells (Fig. 6 C).

DISCUSSION

The results of the present study indicate that treatment with FK506 improves the success of regeneration and the degree of functional recovery after long gap nerve injury repaired with artificial nerve guides. With a simple collagen guide, nerve regeneration was clearly improved by coadjuvant administration of FK506. Moreover, when the collagen guide was seeded with allogeneic SCs, FK506 also enhanced the progress of nerve regeneration. Thus, combination of collagen guides seeded with SCs and administration of FK506 enables successful regeneration and recovery after a 6-mm gap injury in the mouse sciatic nerve. Such a distance is considered to be limiting for axonal regeneration through silicone tubes (Butí et al., 1996; Gómez et al., 1996; Navarro et al., 1996) and is only effectively bridged in a

Fig 5. Cross-sections of regenerated nerves at the mid-point of the collagen guide four months following sciatic nerve resection and repair. Representative samples of (A) group C+FK506, (B) group M, (C) group SC and (D) group SC+FK506. Note the presence of regenerated fibers of smaller than normal caliber grouped in mini-fascicles. Bar = 10 μm.

Fig. 6. Longitudinal sections of the regenerated nerve cable at the middle of the collagen guide one month following implantation of a collagen guide seeded with prelabeled SCs, in an untreated animal (A) and in an animal treated with 5 mg/kg FK506 (B). Note the higher density of fluorescent cells in the animal treated with FK506. (C) Regenerative cable immunostained against S-100. The location of positive S-100 cells coincides with that observed in the sections for PKH26-labeled SCs. Bar = 150 μm.

low proportion of cases with resorbable guides, such as those of collagen or polylactide-caprolactone polymer (Navarro et al., 1996).

In regard to the dose of FK506 administered, the 5 mg/kg dosage was chosen because it is the dose most effective in enhancing the rate of axonal regeneration in mice (Udina et al., 2002; 2003a) and in rats (Wang et al., 1997), in addition to this dose being immunosuppressive (Udina et al., 2003b). Immunosuppression was desirable to avoid rejection of the allogeneic donor SCs implanted. Although continuous administration of FK506 has not been shown to be advantageous versus temporal administration in autograft models (Udina et al 2004), we maintained treatment during the entire study in the tubulization model due to the poor results obtained in silicone tubes when FK506 was administrated for only 9 weeks (Navarro et al., 2001), the slower rate of regeneration through nerve guides compared with autologous nerve grafts, and the immune rejection found in allografts after withdrawal of FK506 (Büttemeyer et al 1995, Udina et al 2004).

Autologous nerve grafts, the gold-standard repair method for bridging a gap in peripheral nerves supply a rich environment for regenerative axons, with a population of reactive SCs and endoneurial conduits. In contrast, acellular grafts show a poor capacity to sustain axonal regeneration (Hall et al., 1986b; Gulati et al., 1988), due to the absence of SCs in the graft and the limited capacity of host SCs to migrate in the graft (Hall et al., 1986a,b). Similarly, artificial nerve guides sustain regeneration only through relatively short gaps and do not achieve the same outcome that is possible with autografts when used to repair gaps longer than a critical species-dependent length (Lundborg et al., 1982; Archibald et al., 1995; Butí et al., 1996; Rodríguez et al., 2000; Valero-Cabré et al., 2003). Since artificial guides are initially empty, axonal regeneration depends on the formation of a new matrix scaffold, over which fibroblasts and SCs can migrate from both nerve stumps. This structure allows regenerating axons to elongate from the proximal stump through the guide (Williams et al, 1983). As the gap between the stumps lengthens, the more difficult it is to form the initial connective cable and, subsequently, axonal regeneration is poorer.

Effects of FK506 on repair by collagen guides

FK506 is able to accelerate nerve regeneration and increase the degree of target organ reinnervation (Gold et al., 1994; 1995; Udina et al., 2002, 2003a), but is not able to overcome the failure of regeneration in a poor environment, such as in chronically denervated nerves (Sulaiman et al., 2002) and in long nerve gaps repaired with silicone tubes (Navarro et al 2001). Thus, the drug does not appear able to compensate for the loss of a permissive environment for axonal regeneration. The poor rate of successful regeneration through a 6-mm long gap in the mouse sciatic nerve repaired with silicone tube (only 20%) was not increased by temporal (Navarro et al., 2001) or continuous administration of FK506 (unpublished observations). However, the few FK506-treated animals that did show

regeneration exhibited better functional recovery than the animals with regeneration in the untreated group.

The physical characteristics of the tubes used are determinants for the success of regeneration. Resorbable and permeable guides, such as those made of collagen and polylactide-caprolactone polymers, improve recovery when compared to silicone tubes (Chamberlain et a., 1998; Navarro et al., 1996; Valeró-Cabré et al., 2003). This is likely due to their capacity to allow extraneural supply of nutrients to the regenerating axons, to enhance the formation of the initial matrix and the migration of cells into the guides, all playing a critical role in the success of bridging long nerve gaps (Williams et al., 1987). Our results, in agreement with previous reports (Gómez et al 1996, Navarro et al 1996, Chamberlain 1998), indicate that regeneration within collagen guides occurred in less than half of the animals, a limited albeit higher proportion than in silicone tubes (Butí et al 1996, Navarro et al 2001). Administration of FK506 caused a notable improvement in the number of regenerated nerve fibers and in the degree of functional recovery achieved. A preliminary report demonstrated that FK506 also accelerated nerve regeneration in collagen guides implanted to bridge a 10-mm gap in the rat (Archibald et al 1999).

Effects of FK506 in collagen guides seeded with Schwann cells

When collagen guides were prefilled with Matrigel alone or seeded with SCs functional reinnervation improved only slightly with respect to collagen guides with saline solution, despite our finding that the numbers of myelinated regenerated fibers in the distal nerve was increased by 2-3 times. In the absence of immunosuppressive treatment, transplantation of allogeneic SCs did not demonstrate a clear capacity to enhance regeneration, as indicated by the similar results obtained in the group where the collagen guide was filled only with Matrigel, which is in agreement with previous results (Rodríguez et al., 2000). Reinnervation of distal targets began between 90 and 120 dpo in the majority of the animals in groups M and SC; therefore, a relatively long-term study needs to be undertaken with tubular prostheses to repair long nerve gaps. It is worth noting that, although syngeneic SCs have been shown to survive after implantation at an unknown proportion, a number of the transplanted cells die because of host rejection (Keeley et al 1993, Kim et al 1994, Levi et al 1997). Thus, a high number of allogeneic SCs need to be transplanted initially to ensure that a sufficient number to promote regeneration remains viable during the first weeks after implantation (Ansselin et al 1997).

The administration of FK506 to the mice implanted with collagen guides containing allogeneic SCs (group SC+FK506) resulted in successful regeneration in all the mice and significantly higher levels of target reinnervation compared to untreated groups with collagen guides alone and, for some functions, with collagen guides prefilled with Matrigel and SCs. Here, the additional immunosuppressive action of FK506 avoided rejection of the foreign SCs and enabled the

transplanted reactive SCs to fully exert their proregenerative actions.

Possible mechanisms of action

It is well known that FK506 exerts its immunosuppressive effect through binding to the immunophilin FKBP-12 and calcineurin inhibition (Shreiber, 1991). This immunosuppressive action avoids host rejection of nerve allografts (Büttemeyer et al 1995, Udina et al 2003b) and of allotransplanted SCs. Nevertheless, the neurotrophic action of FK506 is independent of FKBP-12 binding and appears to be dependent upon binding to FKBP-52 (Gold et al., 1999, Gold 2000; Gold and Villafranca, 2003; Gold et al., 2004). FKBP-52 is associated to the mature steroid receptor complex; by binding FK506, the receptor is dissociated and activates several downstream effectors that lead to enhancement of axonal growth, including stimulation of the extracellular signal regulated kinase (ERK) pathway (Gold and Zhong, 2004) and increased expression of c-jun (Gold et al., 1999) and GAP-43 (Gold et al 1998). The acceleration of the rate of axonal regeneration by FK506 is likely due to a direct effect on the axotomized neurons to speed elongation (Wang et al 1997). This mechanism would explain the improved regeneration found in collagen guides treated with FK506. The combination of both immunosuppressive and neurotrophic actions probably underlies the marked improvement in regeneration found in our study for the group transplanted with SCs and given FK506. However, a possible additional action on SCs may also be considered. GAP-43 plays a critical role in axonal growth and regeneration (Benowitz and Routtenberg, 1997) and, in addition, GAP-43 is also expressed in SCs early after nerve injury (Curtis et al., 1992) and it could be involved in motility and elongation of SCs during regeneration (Hall et al 1992). NGF has been proposed as a possible modulator of SC migration (Anton et al., 1994), produced in an autocrine manner by denervated SCs (Heumann et al., 1987). In this context, cross-talk between FK506 and NGF signal transduction pathways via ERK activation has recently been demonstrated (Gold and Zhong, 2004). Further in vitro and in vivo studies are needed to elucidate the possible effects of FK506 on inducing pro-regenerative responses by SCs.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministerio de Ciencia y Tecnología (SAF2002-04016) and the Acadèmia de Ciències Mèdiques de Catalunya i Balears, Spain, and from the EC (IST-2001-35094). We thank Fujisawa Pharmaceuticals Inc. (Osaka, Japan) for its generous gift of FK506, and Integra LifeSciences Corp. (Plainsboro, NJ) for providing the collagen guides. The technical help of Jessica Jaramillo is greatly acknowledged.

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ANNEX

LAS INMUNOFILINAS: AGENTES NEUROPROTECTORES Y PROMOTORES DE LA REGENERACIÓN NEURAL

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RESUMEN

Las inmunofilinas son una familia de proteínas principalmente conocidas porque actúan como receptores de los fármacos inmunosupresores ciclosporina A (CsA) y FK506. Las inmunofilinas ejercen diversas funciones generales, incluyendo las de regular la permeabilidad mitocondrial, modular la estabilidad de canales iónicos y actuar como chaperonas para una variedad de proteínas. Sin embargo, las inmunofilinas se encuentran en particular abundancia en el sistema nervioso. La CsA, el FK506 y otros derivados inhiben la función de las inmunofilinas y, a través del bloqueo o activación de diversos procesos intracelulares, se ha demostrado que ejercen efectos neuroprotectores en distintos modelos experimentales de isquemia cerebral, enfermedad de Parkinson y lesiones excitotóxicas. Asimismo, el FK506 también tiene efectos neuroregeneradores, acelerando la velocidad de regeneración axonal tras lesiones del sistema nervioso periférico. El desarrollo farmacológico de nuevos agentes ligandos selectivos de inmunofilinas ofrece nuevas perspectivas de interés en el tratamiento de procesos degenerativos y lesiones del sistema nervioso.

Palabras clave: inmunofilinas, ciclosporina, FK506, regeneración axonal, neuroprotección.

IMMUNOPHILINS: NEUROPROTECTIVE AGENTS AND PROMOTERS OF NEURAL REGENERATION

SUMMARY

Immunophilins are a family of proteins mainly known because they act as receptors of the immunosuppressant drugs cyclosporin A (CsA) and FK506. Immunophilins serve several general functions, including regulation of mitochondrial permeability, modulation of ion channels stability and acting as chaperones for a variety of proteins. However, immunophilins are also present at high density in the nervous system. CsA, FK506 and other derivatives inhibit the function of immunophilins and, through bloking or activating several intracellular pathways, it has been shown that they exert neuroprotective effects in different experimental models of ischemia, Parkinson's disease and excitotoxic insults. Moreover, FK506 also has neuroregenerative effects, by enhancing the axonal regeneration rate after lesions of the peripheral nervous system. The development of new agents that selectively bind to immunophilins opens new interesting perspectives for the therapy of degenerative diseases and injuries of the nervous system.

Keywords: immunophilins, cyclosporin, FK506, axonal regeneration, neuroprotection.

INTRODUCCIÓN

El descubrimiento de la ciclosporina A (CsA), un péptido cíclico derivado fúngico, revolucionó el transplante de órganos sólidos, ya que supuso una alternativa mucho menos tóxica a los fármacos inmunosupresores citotóxicos. Posteriormente se aisló el tacrolimus o FK506, fármaco con actividad inmunosupresora parecida a la de la CsA pero más potente que ésta. Más reciente ha sido el descubrimiento de la rapamicina o sirolimus que, como el FK506, deriva de un *Streptomices* y es un macrólido. Los tres compuestos citados se unen a unas proteínas intracelulares, las inmunofilinas, formando un complejo a través del cual ejercen su acción farmacológica [1, 2]. Si bien la CsA y el FK506 no tienen homologías estructurales y sus receptores son diferentes, ambos ejercen su acción inmunosupresora al mismo nivel, inhibiendo la calcineurina, una fosfatasa dependiente de calcio. En cambio, la rapamicina, aunque comparte receptor y homologías con el FK506, desencadena la inmunosupresión inhibiendo un paso posterior independiente de calcio. Los tres fármacos, de todas maneras, bloquean la misma respuesta: la proliferación de los linfocitos T estimulada por antígenos específicos o por células alogénicas [1]. Tanto la CsA como el FK506 han sido ampliamente utilizados en la prevención del rechazo de los transplantes de órganos sólidos. A pesar de que la experiencia con el FK506 no es aún tan amplia como con la CsA, el FK506 tiene una potencia inmunosupresora unas 10 veces superior que la CsA, disminuye el porcentaje de rechazos agudos respecto a la CsA y es una buena alternativa como terapia de rescate en rechazos resistentes a las pautas convencionales [3].

Paralelamente a su acción inmunosupresora, el FK506 y otros productos análogos han mostrado propiedades neuroprotectoras y promotoras de la regeneración axonal en diversos estudios experimentales [4, 5], lo que ha convertido a los fármacos ligandos de inmunofilinas en candidatos de interés reciente para mejorar la regeneración nerviosa después de traumatismos y para proteger o limitar la pérdida de neuronas en enfermedades neurodegenerativas o en cuadros de isquemia cerebral.

LAS INMUNOFILINAS

Las inmunofilinas son una familia de proteínas citoplasmáticas que poseen en común una misma actividad enzimática: catalizar la interconversión *cis-trans* de los isómeros de los enlaces amida adyacentes a los residuos prolina de los sustratos peptídicos. Esta actividad catalítica se conoce como prolil-peptidil isomerasa (PPIasa) o rotamasa [6] y fue descrita en paralelo al descubrimiento del receptor que media la acción de la CsA, la ciclofilina (CyP) [7]. Posteriormente se vio que ambas proteínas, enzima y receptor, eran estructuralmente idénticas [8, 9]. Consecuentemente se describió el receptor del FK506, denominado FKBP (*FK binding protein*) y que también tiene la propiedad rotamásica [10], aunque no posee homología con la CyP [11]. Las inmunofilinas son, de hecho, una amplia familia, que se subdivide en FKBPs y CyPs (Tabla 1) dependiendo de su

capacidad de unión al FK506 o a la CsA respectivamente [12, 13, 14]. Aunque inicialmente se relacionó a las inmunofilinas directamente con el sistema inmunitario, por ser receptores de fármacos inmunosupresores, posteriormente se ha visto que los niveles de FKBP12 en el tejido nervioso son considerablemente superiores a los presentes en tejidos del sistema inmune [15]. Este hecho y el que varios estudios hayan otorgado propiedades neuroprotectoras y neurotróficas al FK506 y otros fármacos derivados sugiere que las inmunofilinas juegan un papel fisiológico relevante en el sistema nervioso.

Papel biológico de las inmunofilinas

La familia de las inmunofilinas está muy conservada a lo largo de la evolución, por lo que se supone que estas proteínas deben ejercer un papel relevante en los organismos, pero al ser tan ubicuas y numerosas se les ha implicado en múltiples procesos (Tabla 2). La importancia de las inmunofilinas parece radicar en su habilidad para actuar en condiciones de estrés celular [12].

Ya que algunas inmunofilinas median la acción de fármacos inmunosupresores cabría pensar que existieran ligandos endógenos que actuaran al mismo nivel. Sin embargo, se sabe que es el complejo fármaco-inmunofilina el responsable de desencadenar la acción intracelular, de manera que la geometría de este complejo sería más determinante que no el tipo de inmunofilina implicada. El hecho de que el FK506 y la rapamicina tengan un mecanismo de acción diferente aun cuando actúan sobre la misma inmunofilina receptora (FKBP) y en la misma célula, sugiere que la geometría del complejo inmunofilina-ligando es determinante en la asociación de éste a una vía de señal intracelular específica, por lo que se propone que las inmunofilinas podrían actuar también como presentadoras de moléculas [1]. Las funciones de las inmunofilinas serían así bastante generales, como el doblamiento de proteínas, el transporte de proteínas o la formación de complejos proteicos, y solo cuando se unen a ligandos exógenos específicos serían capaces de interferir en cascadas de señales intracelulares. Así, inmunofilinas como la FKBP12 o la CyPA solamente inhiben a la calcineurina cuando forman complejo con el FK506 o la CsA respectivamente. Debido a que la isomerización de los enlaces amida de los radicales prolil de los péptidos es un paso lento y probablemente limitante en el doblamiento proteico [16], es probable que las inmunofilinas, mediante su actividad rotamásica, regulen este paso y, por tanto, sean determinantes en el doblamiento de algunas proteínas.

Otra acción fisiológica de la rotamasa es la de intervenir en la estabilización y conformación de proteínas de membrana. La FKBP12 interacciona con dos canales iónicos, el receptor de la rianodina (RyR) y el receptor 1,4,5-trifosfato de inositol (IP3R). Ambos son canales de calcio, por lo que esta inmunofilina podría estar implicada en la regulación de la liberación intracelular de dicho ion [13, 17]. El RyR es el principal canal de liberación de calcio localizado en la cisterna terminal del retículo sarcoplasmático, implicado en el proceso de contracción-excitación del

músculo estriado, aunque también se localiza en corazón y cerebro. Su asociación con la FKBP12 le confiere estabilidad tanto en estado abierto como cerrado, por lo que, aunque dificulta su apertura, una vez abierto optimiza el flujo iónico [18]. Por otro lado, el IP3R media la liberación de calcio intracelular desencadenada por hormonas y neurotransmisores, al convertir el fosfatidilinositol fosfato a inositol trifosfato y diacilglicerol [19, 20]. La FKBP12 se une al canal de calcio, le da estabilidad [21] y parece actuar como puente de enlace entre éste y la calcineurina, que se une al complejo y modula el flujo iónico controlando la fosforilación del canal [22]. En cuanto a las ciclofilinas, la CyPD es una proteína de la matriz mitocondrial que también se encuentra asociada a un canal voltaje dependiente y a una translocasa de nucleótidos de adenina. Este complejo en condiciones patológicas forma el MPTP (*mitochondrial permeability transition pore*). Cuando hay un aumento de calcio, una depleción de ATP o un estado oxidativo, el canal se activa y libera calcio al exterior, acompañado de un desarreglo en la cadena respiratoria que provoca que el ATP se hidrolice en vez de formarse. La activación de este canal se ha implicado en la muerte celular inducida por el calcio [23].

Por otro lado, la FKBP12 se une al receptor tipo I del TGFβ (*transforming growth factor* β) e inhibe su capacidad de transducir la señal intracelular [24]. El receptor del TGFβ está compuesto por dos subunidades, la I y la II. La segunda se puede unir al ligando pero necesita interaccionar con el receptor tipo I, al que activa mediante fosforilación, para que se desencadene una respuesta intracelular. Esta fosforilación de la subunidad I comporta la liberación de la FKBP12. Se ha propuesto que la inmunofilina no inhibiría al receptor directamente sino gracias a su capacidad de atraer la fosfatasa calcineurina que, desfosforilando al receptor, lo mantendría inactivo [25].

Además, las inmunofilinas forman parte de algunos complejos proteicos de múltiples subunidades y podrían jugar un papel importante en la compactación y modulación de éstos. En estos casos no parece que la actividad rotamasa medie su acción [14]. La CyP40 y la FKBP52 (o *heat shok protein*, hsp-52), ambas de elevado peso molecular, están asociadas a los heterocomplejos de los receptores de esteroides. La FKBP52 se encuentra asociada a los receptores de esteroides sexuales y al de glucocorticoides, mientras que la CyP40 sólo se ha relacionada con los receptores de glucocorticoide y progestágeno. Estos heterocomplejos están, a su vez, formados por heat shock proteins (hsp), cuya principal función es facilitar el doblamiento del dominio de unión a la hormona. La hsp-90 asociada a la hsp-60 se une al receptor esteroide a través de su dominio de unión a la hormona y favorecen una elevada afinidad para la hormona. La hsp-60 se libera y a la hsp-90 se le asocia la p23, formando un complejo estable conocido como heterocomplejo del receptor no transformado [26]. El dominio de unión de la hsp-90 para la hsp-60 queda libre y es en él donde puede unirse una inmunofilina [27]. El heterocomplejo es muy dinámico y se encuentra en un ciclo constante de ensamblaje y desensamblaje que podría estar implicado en su transporte

citoplasma-núcleo. La FKBP52 parece estar implicada en este movimiento. La unión de la hormona esteroide con el receptor provoca su transformación, se disocia del resto del complejo y adquiere capacidad de unirse al DNA [26].

INMUNOFILINAS E INMUNOSUPRESIÓN

En el sistema inmunitario, la CsA y el FK506 suprimen la inmunidad celular mediante el bloqueo de la proliferación de los linfocitos T, reduciendo la expresión de interleucina 2 (IL-2). También inhiben la expresión de otras citocinas como IL-3, IL-4, IL-5, IFN-g, TNF-α y GM-CSF, producidas por los linfocitos T activados. Aunque se consideran inmunosupresores selectivos de la respuesta celular, también son capaces de alterar la actividad de los linfocitos B y la de los granulocitos [2, 28]. El FK506 también inhibe parcialmente la secreción de IL-1 y la producción de TNF-α en monocitos y macrófagos, aunque a concentraciones muy superiores a las requeridas para inhibir la proliferación de linfocitos T [29, 30].

La CsA y el FK506 actúan como inmunosupresores mediante el mismo mecanismo de acción (Fig. 1): la inhibición de la calcineurina, una fosfatasa que es calcio/calmodulina dependiente [31, 32]. Los antígenos extraños mostrados en la superficie de células presentadoras de antígenos activan los receptores de las células T, iniciando una vía que produce un aumento de calcio al medio intracelular. Este calcio se une a la calmodulina y a la calcineurina B, que activan la actividad fosfatasa de la subunidad catalítica de calcineurina (o calcineurina A). La fosfatasa, a su vez, desfosforila el componente citoplasmático del factor de transcripción nuclear de las células T (NF-AT), permitiendo que pueda translocarse al núcleo, donde se unirá al componente nuclear del NF-AT y promoverá la transcripción del gen de la IL-2 y del receptor de IL-2 [13, 33]. La CyPA y la FKBP12 son las dos inmunofilinas más abundantes en los linfocitos T, por lo que se consideran los principales receptores de las acciones inmunosupresoras de CsA y FK506 respectivamente [1]. El complejo formado por la CsA o el FK506 con su correspondiente inmunofilina, al inhibir la calcineurina, no permite la desfosforilación del componente citoplasmático del NF-AT (Fig. 1), de manera que éste no podrá translocarse al núcleo, por lo que se bloqueará la síntesis de IL-2 [34]. La potencia relativa de la CsA, el FK506 y otros fármacos para inhibir la calcineurina guarda relación con su potencia relativa como inmunosupresor. Parece claro que la inmunosupresión causada por CsA y FK506 está mediada principalmente por la inhibición de la calcineurina, aunque otros factores reguladores de la transcripción del gen de la IL-2 (tales como NF-κB y Oct-1/OAP) también podrían estar implicados [34]. Por otra parte, aunque la FKBP12 es la principal proteína responsable de la inmunosupresión desencadenada por el FK506, más recientemente se ha demostrado que la FKBP51, específicamente expresada en células T, también inhibe a la

calcineurina [35], sugiriendo que diversas inmunofilinas pueden mediar las acciones inmunosupresoras.

Por su parte, la rapamicina, a pesar de ser estructuralmente análoga al FK506, de compartir los receptores FKBP y de inhibir también su actividad rotamásica de forma potente, actúa como inmunosupresor bloqueando un paso más tardío, no dependiente de calcio, de la estimulación de las células T [1, 36]. El complejo rapamicina-FKBP12 interacciona con la proteína RAFT (*rapamycin and FKBP target*), inhibiendo la fosforilación de la p70-S6-cinasa que fosforila la proteína ribosomal S6 (Fig. 1), con lo que se bloquea la transición de los linfocitos T de la fase G1 a la fase S del ciclo celular [37]. Además, la rapamicina inhibe la fosforilación de una proteína reguladora del factor de iniciación de la translación (la eIF4E). La rapamicina, pues, induce inmunosupresión al inhibir la proliferación de clones de células T estimulada por la IL-2.

Mediante la utilización del 506BD, una pequeña molécula que posee el dominio de unión para ligarse con elevada afinidad a la FKBP e inhibir su actividad rotamásica, se descartó que esta inhibición estuviera implicada en la acción inmunosupresora del FK506. Este ligando no tenía ningún efecto sobre la activación de las células T, aunque sí bloqueaba la acción de los otros fármacos al ser administrada conjuntamente [38]. Estos datos no sólo permiten deducir que la inhibición de la rotamasa no es suficiente para explicar los efectos del FK506 y la CsA sobre los linfocitos T, sino que apoyan la noción de que estos fármacos tienen dos dominios, uno que se une a la inmunofilina (dominio de unión) y otro responsable de mediar las acciones biológicas del complejo inmunofilina-fármaco (dominio efector) [1, 14]. Además, como los inmunosupresores actúan a concentraciones insuficientes para bloquear la actividad rotamásica por completo, su acción no debe ser la inhibición de las inmunofilinas, sino incrementar su función, induciendo a las inmunofilinas a interactuar con otros blancos.

INMUNOFILINAS EN EL SISTEMA NERVIOSO

La concentración de las inmunofilinas en el cerebro es mucho mayor que en el tejido inmunitario, como el timo, y más aún que en diversos tejidos periféricos, como bazo, hígado, riñón o pulmones [15]. La localización regional del FKBP12 presenta una distribución paralela a la de la calcineurina, con altas concentraciones especialmente en córtex, hipocampo, ganglios basales y cerebelo [39]. También existe una similar localización de calcineurina y ciclofilina. Esta relación topográfica sugiere que existe una relación funcional entre las inmunofilinas y la calcineurina; la unión de CsA a CyP o de FK506 a FKBP bloquea la actividad de la calcineurina, regulando así la acción de una variedad de fosfoproteínas fosforiladas a nivel neuronal. El papel de las inmunofilinas mediado por inhibición de calcineurina intervendría en la liberación de calcio intracelular, en los procesos de depresión a largo plazo en el hipocampo, en la desensibilización de

receptores NMDA, en el control de la liberación de neurotransmisores y en la regulación de la sintasa de óxido nítrico (NOS), entre otros procesos.

La calcineurina se ha identificado como un componente clave en la transición de la memoria de corto plazo a largo plazo, así como elemento regulador de los procesos de potenciación (LTP) y de depresión (LTD) a largo plazo. Se ha demostrado que el FK506 facilita la LTP en el hipocampo e inhibe la LTD en el hipocampo y en el córtex visual [40]. Dado que la génesis de LTP i LTD requiere de la formación de NO, los efectos del FK506 en este aspecto serían atribuibles a la inhibición de la activación de la NOS dependiente de calcineurina. En cultivos de células PC12 y en preparaciones de sinaptosomas se ha demostrado que FK506 y CsA también producen una inhibición de la liberación de neurotransmisores inducida por NMDA. Este efecto es explicable por la inhibición de la producción de NO, ya que la inhibición de la calcineurina mantiene la NOS en un estado fosforilado inactivo [41, 42]. Por el contrario, el FK506 estimula la liberación de neurotransmisores, tanto espontánea como inducida por despolarización, en sinaptosomas, acción que parece debida a una hiperfosforilación de proteínas de las vesículas sinápticas, como sinapsina-1 [42].

Sin embargo, el principal interés del estudio de las inmunofilinas y sus fármacos ligandos en el sistema nervioso ha surgido a partir de las evidencias halladas en estudios experimentales, tanto in vitro como in vivo, de que el FK506 así como otros análogos tienen propiedades neurotróficas y neuroprotectoras. En clínica humana no hay aún estudios específicos para evaluar estas propiedades, pero sí se han encontrado indicios indirectos en los estudios que valoran su potencial inmunosupresor. En un heterotransplante de mano, cuya terapia inmunosupresora incluía la administración sistémica de FK506, se observó una recuperación más rápida de lo esperado de las funciones sensorio-motoras, lo que sugería una buena regeneración de los nervios suturados, aunque no se realizó ningún test funcional para corroborar esta impresión [43]. Por otro lado, en un estudio comparativo entre la CsA y el FK506 en pacientes con transplante hepático, se observó una elevada incidencia de isquemia cerebral global en los inmunosuprimidos con CsA (50%), mientras ninguno de los tratados con FK506 presentó esta complicación. Como el FK506 atraviesa bien la barrera hematoencefálica, al contrario que la CsA, se postuló que el FK506 podría jugar un papel neuroprotector a nivel cerebral [44], aunque no puede descartarse un efecto tóxico de la CsA. Por el contrario, se han descrito algunos casos de leucoencefalopatía relacionada con la inmunosupresión en los pacientes tratados tanto con FK506 como con CsA. Reduciendo la dosis de los fármacos la clínica revierte espontáneamente, hecho que sugiere que la inhibición de la calcineurina por parte de estos compuestos podría provocar desmielinización [45].

INMUNOFILINAS Y NEUROPROTECCIÓN

Los descubrimientos de que las inmunofilinas se expresan en el sistema nervioso a alta densidad y colocalizadas con la calcineurina, y de que la NOS neuronal es una diana de la calcineurina, condujeron a investigar si los fármacos ligandos de inmunofilinas podrían ejercer neuroprotección ante diversos tipos de agresiones. In vitro, un primer estudio describió que en cultivos de neuronas corticales la adición en el medio tanto de FK506 como de CsA inhibía la neurotoxicidad inducida por NMDA, aunque no alteraba la mediada por kainato [41]. Posteriormente, en un modelo animal de oclusión de la arteria cerebral media, se demostró que la administración intravenosa de FK506 (1 mg/kg un minuto después de la oclusión) reducía el volumen de tejido dañado. Por el contrario, la misma dosis de CsA o de rapamicina no mostraban ningún efecto neuroprotector [46]. No obstante, en otro estudio más reciente y utilizando una dosis mayor de CsA (5 mg/kg) sí que se observó una reducción de la lesión [47]. Aunque en un principio se relacionó la capacidad antiexcitotóxica del FK506 con su efecto neuroprotector en la isquemia, el estudio del mismo equipo descartó este mecanismo, ya que cuando la lesión cerebral era inducida por un tóxico cuyo efecto es bloqueado por un antagonista del receptor NMDA, ni el FK506 ni la CsA mostraban efecto neuroprotector [47]. De todas maneras, el potencial neuroprotector de la CsA sigue en duda, porque en otros estudios, éste fármaco no ha evidenciado ningún efecto protector en neuronas hipocampales y corticales ante la isquemia transitoria global, mientras que el FK506 mostró un claro efecto protector [48]. Por otro lado, hay que tener en cuenta que en los modelos de isquemia cerebral donde no se produce una disrupción de la barrera hematoencefálica, la falta de neuroprotección de la CsA podría explicarse por su pobre permeabilidad [49]. Esta limitada permeabilidad puede ser contrarrestada utilizando dosis mucho más altas o si hay ruptura de la barrera. Así, la CsA se mostró neuroprotectora del daño provocado por la isquemia transitoria en ratas si su administración sistémica se combinaba con una lesión intracerebral [50, 51].

Por el contrario, en un estudio de isquemia inducido por hipoglicemia severa, la administración previa de una única dosis de FK506 (2 mg/kg i.v.) no se mostró neuroprotectora, a diferencia de la CsA (50 mg/kg i.v.) que disminuía el daño celular [52]. En un modelo de contusión cerebral, tampoco el FK506 mostró efectividad, mientras que la CsA disminuyó significativamente el volumen de tejido dañado respecto al grupo control [53]. La dosis neuroprotectora más efectiva en este modelo fue la de 20 mg/kg, administrada 15 minutos y 24 horas después de la lesión, mientras que dosis menores o superiores resultaron algo menos efectivas [54]. En la patogénesis de estos dos modelos se ha implicado la activación de canales de permeabilidad a nivel mitocondrial ante el estrés oxidativo [23]. La ineficacia del FK506 podría explicarse por la falta de interacción con inmunofilinas mitocondriales. La CsA, en cambio, bloquearía la apertura de estos canales mediante su unión a una ciclofilina mitocondrial, la CyPD [55] (Fig. 2).

En cuanto a modelos experimentales de lesión medular, la administración de CsA, con la misma dosis (20 mg/kg i.p. seguida de infusión continua de 2,5 mg/kg) que se había demostrado

efectiva en el traumatismo cerebral, no produce efectos beneficiosos, ni en la recuperación funcional ni en el volumen de la lesión, tras contusión de la médula espinal [56]. Por el contrario, después de lesión de los cordones posteriores de la médula espinal, la administración sistémica de 0,5 o de 2 mg/kg de FK506 promueve una mayor supervivencia de los axones no lesionados respecto a un grupo control [57]. En un modelo de foto-trombosis de la médula espinal también se ha visto que la administración de dosis muy bajas de FK506 (0,25 mg/kg) durante los 3 primeros días mejora la recuperación funcional de los animales [58], mientras que la CsA no induce efectos apreciables [59].

También se han utilizado modelos experimentales de neurodegeneración para evaluar los efectos de estos fármacos. Así, en el modelo de Parkinson inducido por el tóxico MPTP, la administración de FK506 ha mostrado efectos neuroprotectores [60], aunque estos resultados no se han confirmado en un estudio posterior [61], en el que si bien el FK506 no se mostraba efectivo para proteger las neuronas estriatales, un análogo sin efecto inmunosupresor, el V-10.367, sí que lo era. Estudios con otros análogos del FK506 han ofrecido resultados parecidos en el mismo modelo de Parkinson; la administración concomitante de ligandos de FKBPs con el tóxico disminuye el porcentaje de neuronas dañadas y su administración posterior incrementa la regeneración de neuronas dopaminérgicas [14]. Estudios sobre cultivos de neuronas dopaminérgicas también han corroborado que la adición de FK506 o de otros ligandos de inmunofilinas no-inmunosupresores promueve la supervivencia y la extensión de neuritas de dichas neuronas ante la toxicidad de MPP^+ o de 6-OH-dopamina [62, 63].

En cuanto a los posibles mecanismos de acción (Fig. 2), como el exceso de NO se ha implicado en la muerte neuronal por excitotoxicidad, la inhibición de la NOS por bloqueo de la calcineurina fue la primera hipótesis del efecto protector del FK506 y de la CsA en lesiones excitotóxicas con exceso de glutamato [41] y en la isquemia cerebral in vivo [13, 46]. Sin embargo, esta opción parece descartada ya que en un modelo in vivo de excitotoxicidad inducido por quinolinato, cuya toxicidad era contrarrestada por un antagonista del receptor NMDA, la administración de FK506 no mostraba ningún efecto protector [47]. Además, en el modelo de isquemia focal por oclusión de la arteria cerebral media en rata, el FK506 ejerce una potente protección, pero no altera la actividad de la NOS medida in vivo [64]. Recientemente se ha postulado que la neuroprotección mediada por FK506 y CsA puede ocurrir mediante la inhibición, mediada por calcineurina, de la desfosforilación de BAD [5, 65]. La calcineurina causa la desfosforilación de BAD, un componente de la familia bcl2, promoviendo la apoptosis. La inactivación y la diana mitocondrial de BAD durante la apoptosis es suprimida por FK506 y por CsA mediante la inhibición de la calcineurina. Sin embargo, en algunos de los modelos ensayados, como el de toxicidad dopaminérgica, se ha demostrado que el FK506 es neuroprotector incluso sobre células de animales *knock-out* para la

FKBP12, lo que apunta a que estos efectos podrían ser mediados por otras inmunofilinas y por mecanismos independientes de la inhibición de la calcineurina [63].

PAPEL NEUROTRÓFICO DE LAS INMUNOFILINAS

Varios estudios han confirmado que la adición de FK506 en cultivos neuronales potencia el crecimiento de neuritas, si bien dentro de un rango de dosis adecuado, ya que en exceso se muestra inhibidor. Así, una dosis de 1-10 nM se considera la más eficaz, mientras que dosis del rango de micromoles inhiben el crecimiento de las neuritas [4, 66, 67]. La curva dosis-respuesta para el FK506 resulta invertida a elevadas dosis, coherente con los datos de experimentos in vivo [68], de lo que se deduce que el FK506 en exceso bloquea su propio efecto neurotrófico. Por su parte, la CsA requiere de dosis mucho más elevadas (50 nM) para ser eficaz en cultivos de neuronas derivadas de la cresta neural (PC12), si bien a dosis de 1 µM tampoco se muestra efectiva para estimular la neuritogénesis de una línea celular derivada del neuroblastoma humano (SH-SY5Y) [4]. La rapamicina también ejerce efectos neurotróficos en cultivos de células PC12 y de neuronas del ganglio sensorial en un rango de dosis parecido al del FK506, si bien las neuritas presentan una morfología diferente a las estimuladas con éste último [69]. Un estudio más detallado, utilizando cultivos de neuronas dopaminérgicas, describe que la CsA y el FK506 promueven la elongación de las neuritas, mientras que la rapamicina y otro análogo (el V-10,367) incrementan la arborización. Curiosamente, a dosis muy bajas, cuando el FK506 se muestra ineficaz para incrementar el crecimiento neurítico, potencia también la arborización [62]. Los efectos contradictorios descritos en referencia a la capacidad neurotrófica de la CsA, dependiendo de la línea celular utilizada, podrían ser debidos a la distribución no homogénea de las inmunofilinas [62].

Los tres fármacos, para ser efectivos, requieren de la presencia de NGF (*nerve growth factor*) en cultivos de PC12 [66] o de SH-SY5Y [4]. Sin embargo, en cultivos de neuronas corticales o hipocampales [4] o en cultivos de neuronas de ganglios de la raíz dorsal (DGR) [69] no es necesaria la presencia de ninguna neurotrofina para que el FK506 ejerza su acción neurotrófica. No obstante, en los cultivos de DRG, la adición de anticuerpos contra NGF bloquea parcialmente la acción del FK506, de manera que es probable que sea la producción endógena de NGF la que medie la acción de los fármacos en estos casos.

Utilizando dosis más elevadas de FK506 (100 µM) en cultivos de células de Schwann el fármaco promueve significativamente el incremento de la población glial, a la vez que disminuye el número de fibroblastos respecto a un cultivo control [70]. La estimulación de la proliferación glial se ha relacionado con un aumento de los niveles de calcio intracelular. Sin embargo, es difícil relacionar este efecto sobre las células de Schwann con las acciones neuritotrópicas del FK506, que a dosis tan elevadas es incluso inhibidor. La vía de acción del FK506 en este caso podría estar

mediada por la FKBP38, que se expresa en las células de Schwann además de en diversas regiones del sistema nervioso central [71].

INMUNOFILINAS Y REGENERACIÓN AXONAL

Tras una lesión por aplastamiento del nervio ciático, se demostró que la administración sistémica de FK506 (1 mg/kg durante los 18 días del seguimiento) aceleraba la recuperación funcional y que el grupo tratado mostraba una mayor densidad de fibras mielínicas y un grado de mielinización más avanzado [72]. A fin de valorar directamente el crecimiento axonal se utilizaron técnicas de radiomarcaje, observándose que el FK506 incrementaba significativamente la velocidad de regeneración axonal. La curva dosis-respuesta presenta forma de campana; el incremento porcentual de la tasa de regeneración fue de 16, 34 y 29% por encima de los animales no tratados, a dosis de 1, 5 y 10 mg/kg respectivamente [73, 68]. De todas maneras, la dosis de 5 mg/kg es relativamente elevada y en algunos estudios se ha preferido utilizar dosis menores para testar su eficacia y posible aplicación clínica. En el mismo modelo de aplastamiento del nervio tibial de la rata también se ha comprobado que una dosis de 1 mg/kg comporta una recuperación funcional más precoz que en el grupo control [74]. El efecto proregenerador se ha visto que es mayor cuando el FK506 se administra diariamente durante todo el periodo de seguimiento (18 días tras la lesión nerviosa) que durante parte del mismo (9 días) [75]. Por el contrario, la administración de CsA (a dosis entre 5 y 50 mg/kg) no presenta, en ningún momento del seguimiento, diferencias significativas respecto al grupo control [68, 74]. Después de sección y reaproximación por sutura del nervio ciático de la rata, se han obtenido unos resultados similares, ya que el grupo tratado con FK506 mostró una recuperación más precoz, superando al grupo control en 14 días, mientras que el grupo tratado con CsA no mejoraba significativamente respecto al no tratado [76].

Administrados como tratamiento inmunosupresor, tanto el FK506 como la CsA permiten la aceptación de aloinjertos y una efectiva regeneración axonal a través de los mismos, aunque la retirada del tratamiento conduce a una reducción de la progresión de la recuperación y a la aparición de signos de rechazo en el aloinjerto [77, 78]. Sin embargo en lesiones severas de nervios periféricos, los efectos del FK506 para estimular la regeneración no han resultado tan claros. Así, tras la reparación por autoinjerto o por isoinjerto de una resección de 2 cm del nervio ciático en la rata, se ha hallado que la administración de FK506, a dosis entre 0,3 y 1 mg/kg, adelanta el inicio de la recuperación locomotora, aunque los niveles finales alcanzados eran equivalentes a los del grupo control. Histológicamente, el grupo tratado mostraba una mayor densidad de fibras mielínicas regeneradas a mitad del seguimiento, pero que se igualaba a la del grupo control al final del estudio [79, 80]. A las 2 semanas de la lesión y reparación, el grupo tratado mostró, además de un incremento en el número de axones regenerados, una reducción de los restos de mielina, lo que podría interpretarse como una aceleración de la degeneración walleriana y una mayor ramificación

axonal [70]. Utilizando también la reparación por autoinjerto de una resección de 6 mm del nervio ciático de ratón, se ha detectado una aceleración en la recuperación de las diferentes funciones neurales con la administración de FK506 (5 mg/kg durante los dos primeros meses postlesión). El tratamiento con FK506 mejora particularmente las funciones que dependen directamente de la elongación neuronal, como las sensoriales, pero menos las que requieren el restablecimiento de sinapsis con el órgano diana, como las motoras [81].

El tratamiento con FK506 también incrementa el número de axones regenerados a través de una guía de colágeno implantada para reparar una resección de 10 mm en el nervio ciático de la rata [82]. Sin embargo, el FK506 ejerce efectos más limitados en situaciones en que la regeneración está limitada, como en distancias limitantes reparadas por tubulización. Así, tras resección de 6 mm en el nervio ciático del ratón y reparación por tubulización, la proporción de animales con éxito de regeneración axonal fue similarmente baja con o sin administración de FK506, aunque los animales tratados con FK506 presentaron una recuperación funcional más rápida y una mayor maduración de las fibras regeneradas [81].

También se ha testado el efecto de fármacos ligandos de inmunofilinas en secciones de las raíces espinales dorsales. En este modelo la regeneración se ve bloqueada a nivel medular por el ambiente inhibitorio generado a nivel de la interfase glial entre el sistema periférico y el central, que los axones no pueden superar. Utilizando un marcador inmunohistoquímico, el CGRP, se observó que, después de seccionar la raíz dorsal L5, los animales tratados diariamente con FK506 (0,5 mg/kg) o CsA (5 mg/kg) presentaban un mayor número de fibras marcadas que eran capaces de atravesar la barrera astrocitaria y regenerar dentro de la médula, arborizándose más que en los animales controles [83].

Después de la sección de los cordones posteriores de la médula espinal de la rata, se ha ensayado el efecto de la administración sistémica de 0,5 o de 2mg/kg de FK506. Mediante estudio con retrotrazadores se demostró que, en el grupo tratado, unos pocos axones lesionados conseguían crecer, aunque la distancia recorrida era muy corta. Por otro lado, la supervivencia de los axones no lesionados era superior en el grupo tratado, hecho que sugiere que el FK promueve la supervivencia de las neuronas axotomizadas. En este mismo estudio también se testó otro ligando de las FKBP pero sin dominio para unirse a la calcineurina, el GP1-1046, y no se encontró ninguna mejoría en la supervivencia axonal [57]. Por otra parte, se ha descrito que el tratamiento con FK506 promueve la regeneración de fibras del fascículo rubroespinal seccionado a lo largo de un injerto de nervio periférico [84], lo que sugiere que los efectos proregenerativos pueden ser relativamente selectivos para distintas poblaciones neuronales.

MECANISMOS DE ACCIÓN DE LOS FÁRMACOS LIGANDOS DE LAS INMUNOFILINAS EN LA REGENERACIÓN NEURAL

Muchos estudios avalan, pues, las propiedades neurotróficas del FK506, aunque éstas no son compartidas por la CsA. A pesar de que los mecanismos de acción del FK506 en el tejido nervioso lesionado no están completamente esclarecidos, parece que, como en la acción inmunosupresora, la inhibición de la rotamasa no jugaría un papel relevante. Los fármacos administrados alcanzan concentraciones nanomolares en el organismo, mientras que las principales inmunofilinas están a nivel de micromoles, de manera que no es factible que se pueda conseguir una inhibición apreciable de éstas. Por ello se cree que los efectos en el sistema nervioso son debidos a que el compuesto fármaco-inmunofilina resultante desencadena acciones intracelulares mediadas por diversas posibles vías metabólicas [1].

Unión a FKBP12 e inhibición de calcineurina

Ya que el FKBP12 se encuentra a elevadas concentraciones en el tejido nervioso [15] se pensó inicialmente que esta inmunofilina también era la principal mediadora del efecto neurotrófico. Además, se ha detectado un aumento importante de los niveles de RNAm de FKBP12 en neuronas motoras después de la lesión del nervio facial [85]. No obstante, este incremento de expresión no va acompañado de un aumento en la expresión de calcineurina en modelos de lesión nerviosa de ciático o de facial.

Los dos blancos principales de la calcineurina en el tejido nervioso son la NOS y la GAP-43 (*growth associated protein* 43). Sus propiedades podrían explicar los efectos neuroprotectores y neurotróficos del FK506, de manera que la vía FKBP12-calcineurina se consideró una buena candidata como vía de acción de este fármaco en el tejido nervioso. La inhibición de la calcineurina mantiene la NOS en un estado hiperfosforilado que la hace inactiva [41] y, por lo tanto, evita la síntesis de NO. El papel que puede jugar el NO en la regeneración nerviosa es complejo y no está bien definido. La inhibición de la NOS potencia la regeneración nerviosa periférica in vivo [86], pero provoca el colapso de los conos de crecimiento neurales in vitro [87]. Por otro lado, puede que la NOS constitutiva y la inducible jueguen papeles distintos en la regeneración ya que estudios con ratones *knock-out* para esta segunda proteína muestran un retraso en la regeneración nerviosa [88]. La NOS inducible es calcio independiente, de manera que no se vería afectada por la inhibición de la calcineurina.

La GAP-43 juega un papel clave en el crecimiento axonal y en la modulación de nuevas conexiones sinápticas [89]. Se ha descrito un incremento en la expresión de GAP-43 en las neuronas después de axotomía periférica [85, 90], que seguía estrechamente la elevación de FKBP12. Este incremento de GAP-43 es superior en los animales tratados con FK506, tanto en lesiones de nervio ciático [90] como en lesiones espinales [58], aunque el incremento de GAP-43 no se correlaciona con un incremento de la proporción de GAP fosforilada. La GAP-43 se asocia a la calmodulina, unión que evita que la proteína cinasa C (PKC) fosforile la GAP-43. Pero un estímulo intenso de la PKC

puede desplazar la calmodulina, a la vez que fosforilar la GAP-43. La GAP-43 libre fosforilada interactúa con elementos del citoesqueleto y promueve la motilidad del cono terminal, mientras que la calmodulina desencadena otras señales intracelulares, entre ellas la activación de la calcineurina que, a su vez, desfosforila la GAP-43. LA GAP-43 desfosforilada puede unirse de nuevo a la calmodulina, de manera que se autolimita su activación [89]. Así, inhibiendo la calcineurina, se alargaría el periodo de activación de la GAP-43. Sin embargo, parece que la GAP-43 relevante en la elongación de los conos axonales sería la fosforilada por estímulos ambientales, mientras que la GAP-43 activada por otros mecanismos, como la fosforilada por la PKC, podría ser inefectiva [91] o incluso interferir en la regeneración [4].

Sin embargo, en años recientes se han acumulado evidencias en contra de que la principal mediadora del efecto promotor de la regeneración del FK506 sea la FKBP12. Así, en un estudio con neuronas procedentes de ratones *knock-out* para la FKBP12, se comprobó que el crecimiento de neuritas de las células tratadas con FK506 era similar al de las neuronas de la cepa control [92]. Además, de ser un mecanismo dependiente de calcineurina, sería de esperar que la CsA mostrara efectos paralelos al FK506 y que no lo hiciera la rapamicina. Sin embargo, esta última incrementa el crecimiento de neuritas in vitro con una potencia parecida a la del FK506 y antagoniza sus efectos si se administran conjuntamente [13, 61]. Por otro lado, diversos análogos del FK506 que presentan unión a inmunofilinas pero que no inhiben la calcineurina han mostrado efectos neuritotróficos in vitro y aceleran la regeneración in vivo [14, 69, 93].

De todas maneras, debido al gran abanico de efectos sobre el sistema nervioso, es probable que haya diferentes inmunofilinas implicadas y su activación podría implicar tanto mecanismos dependientes como independientes de la calcineurina. [14]. Además, las acciones neuroprotectoras y neurotróficas no tendrían porqué compartir una misma vía de activación. No parece que la inhibición de la calcineurina sea el principal mecanismo implicado en la regeneración e incluso podría ser el responsable de la pérdida de eficacia del FK506 a elevadas dosis, pero podría explicar algunos efectos neuroprotectores del FK506 y la CsA.

Modulación de canales de calcio

El FKBP12 modula el flujo de calcio de los receptores de rianodina y de IP3, de manera que la acción del FK506 y la rapamicina sobre el sistema nervioso podría ser debido a un aumento de calcio intracelular provocado por la disociación del complejo que forma la inmunofilina con su canal.

La inactivación del receptor IP3 en los conos de crecimiento de neuronas de la raíz dorsal comporta la inhibición del crecimiento neurítico [94], por lo que los ligandos de la FKBP, alterando este canal, podrían influenciar en la regeneración. De todas maneras, se necesitan unas concentraciones muy elevadas de FK506 para disociar la unión FKBP-canal, hecho que no encaja con las bajas

concentraciones a las que el fármaco potencia el crecimiento neurítico in vitro. Por otro lado, se sabe que la ramificación y elongación de neuritas sólo se promueven cuando los niveles intracelulares de calcio se mantienen dentro de unos límites permisibles [95]. Por lo tanto, la alteración de estos canales de calcio por las inmunofilinas podría explicar la pérdida de efectividad neurotrófica [68] del FK506 cuando se administra a elevadas dosis.

Activación del receptor del TGF-β

La FKBP12 es un inhibidor natural de las señales mediadas por el TGF-β, con lo que ligandos como el FK506, desplazando a la inmunofilina, activan al receptor del TGF-β [24]. El TGF-β estimula la síntesis de NGF por parte de las células gliales [96], de manera que podría potenciar la regeneración por esta vía. Por otro lado, la adición de TGF-β a cultivos de neuronas dopaminérgicas promueve la supervivencia de éstas [97], con una potencia parecida al GDNF. También se han descrito efectos similares entre el FK506 y el GDNF para promover la elongación de neuritas en cultivos de neuronas dopaminérgicas [61], por lo que podría existir un mecanismo de acción paralelo entre ambos. Sin embargo, si el receptor del TGF-β estuviera implicado en la acción neurotrófica, sería de esperar que la CsA no tuviera un efecto parecido al FK506 y sí que lo tuviera la rapamicina, pero en este mismo estudio, la CsA también promueve la elongación de neuritas, mientras que la rapamicina no estimula la elongación pero sí incrementa la arborización [61]. Además, también se necesitan dosis relativamente elevadas de FK506 para desbloquear el receptor de TGF-β de la FKBP12 [24].

Unión a FKBP52 y activación del complejo receptor esteroidal

Una inmunofilina alternativa a la FKBP12 como mediadora de este efecto neurotrófico es la FKBP52. LA FKBP52 es una *heat shock protein* que, entre otros complejos, se asocia al del receptor glucocorticoide maduro [98]. Diversos estudios sobre el crecimiento de neuritas en cultivos de células SHSY5Y de neuroblastoma humano han aportado evidencias de que el efecto del FK506 y análogos puede implicar a la FKBP52 asociada al receptor esteroidal [99]. La adición a los cultivos de un anticuerpo contra la FKBP52 incrementaba el crecimiento neurítico aun más que la adición de FK506. Administrado conjuntamente, tal anticuerpo bloqueaba el efecto del FK506 y también el del NGF. Este hecho y el que el FK506 incremente la sensibilidad de las células al NGF [66] sugieren que las cascadas de señales intracelulares de neurotrofinas y neuroinmunofilinas pueden converger. Por otra parte, la adición de geldamicina, que activa el complejo receptor sin provocar su disociación, también incrementaba el crecimiento neurítico e inhibía el efecto del FK506 y las hormonas esteroides. Las hormonas esteroides y la geldamicina tienen efectos opuestos en la translocación de los ligandos nucleares al receptor, pero ambos potencian el crecimiento neural, por

lo que parece que el mecanismo neurotrófico no depende de la acción directa de la hormona sobre el núcleo. El molibdato, que evita la disociación del complejo glucocorticoide, reduce el crecimiento neurítico promovido por los diferente componentes, sugiriendo que la disociación del complejo está implicada en el mecanismo de acción neurotrófico.

La hipótesis de las vías de acción mediadas por la FKBP52 (Fig. 3) implica que, cuando la hormona esteroide se une al receptor, el complejo se disocia. La hsp-90 se separa del complejo y se activa, sufriendo un cambio conformacional que libera la unidad p23, hasta entonces unida a la hsp-90. El FK506 se uniría indirectamente a la hsp-90 mediante la FKBP52, pero también provocaría la liberación de la p23. El anticuerpo anti-FKBP52, no sólo liberaría la p23 sino también disociaría la inmunofilina, que podría interactuar con elementos del citoesqueleto y jugar un papel destacado en el transporte axonal y, por lo tanto, en la elongación axonal [92]. Además, la interacción entre hsp-90 activada y la MAP cinasa implica una posible conexión con la vía de transducción de señales de las neurotrofinas y la consiguiente estimulación de mecanismos de crecimiento neural. Finalmente, aunque las hormonas esteroides difunden, en principio, por difusión simple, existen también transportadores implicados en su desplazamiento fuera de la célula. Se sabe que el FK506 inhibe uno de estos transportadores, el Mdr1, hecho que podría explicar que potenciara la acción de algunos esteroides y que revirtiera la resistencia a los corticoides en pacientes transplantados [100].

AGRADECIMIENTOS

Este trabajo ha sido posible gracias a la Beca de investigación básica de la Acadèmia de Ciències Mèdiques de Catalunya i Balears.

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PIES DE FIGURAS

Fig. 1. Mecanismos de acción de los fármacos ligandos de inmunofilinas para producir inmunosupresión. Los complejos CsA-ciclofilina y FK506-FKBP12 inhiben a la calcineurina e impiden la translocación al núcleo del NFAT y la activación de los linfocitos T. La rapamicina (RAPA) se une a la FKBP12 bloqueando la acción de RAFT, con lo que se inhibe la traducción proteica y la progresión de la proliferación de los linfocitos T.

Fig. 2. Mecanismos de acción mediante los cuales los fármacos ligandos de inmunofilinas pueden ejercer efectos neuroprotectores.

Fig. 3. Mecanismos de acción, mediados por la activación del complejo receptor esteroide y la FKBP52, que pueden promover efectos neurotrógicos y la regeneración axonal (modificado de Gold 1999).

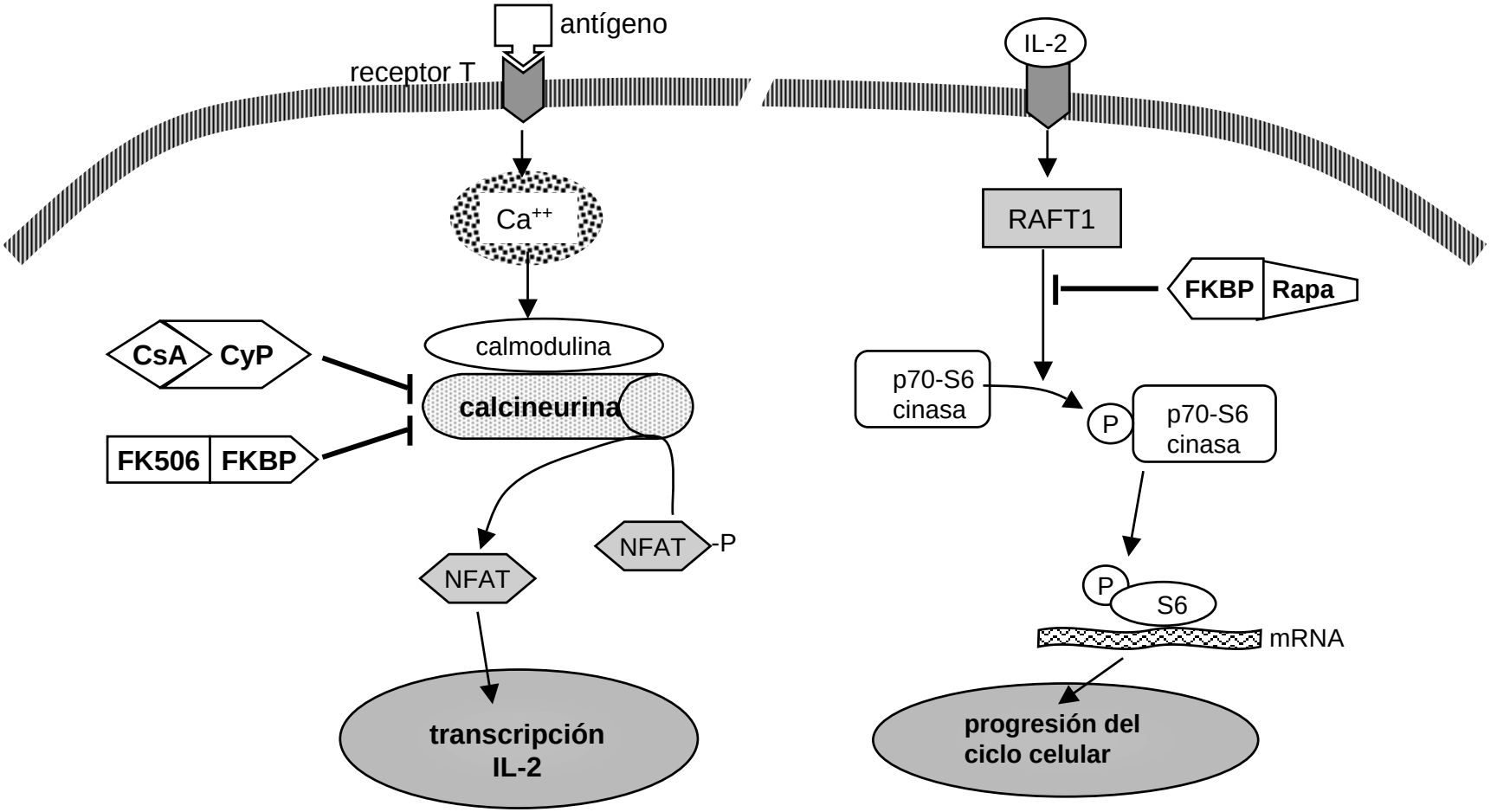
Tabla 1. Inmunofilinas identificadas en tejidos de mamíferos.

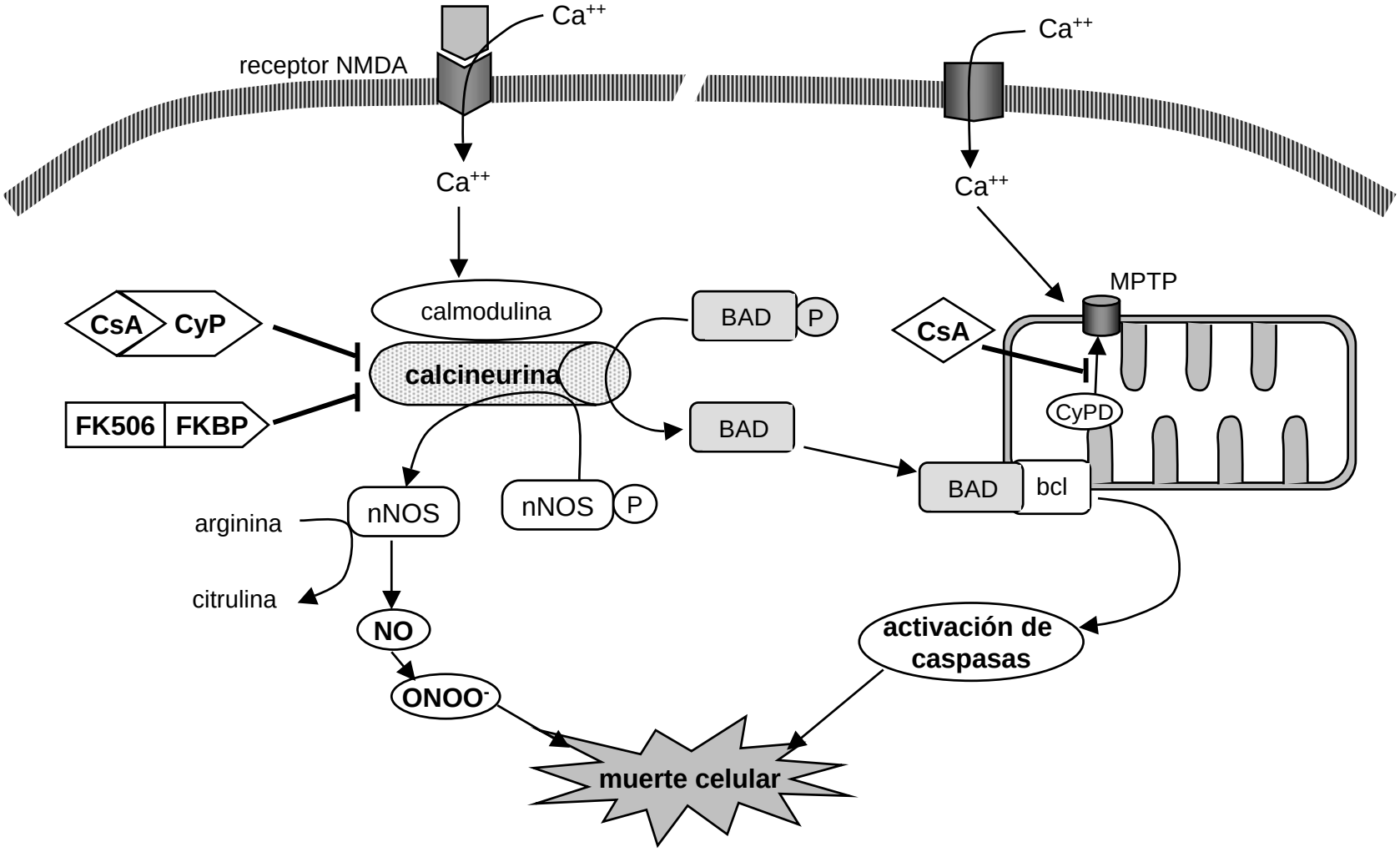
Ciclofilinas	PM	Localización celular	FKBPs	PM	Localización celular
CyP A	18 kDa	citosol	FKBP12	12 kDa	citosol
CyP B	23 kDa	retículo endoplásmico	FKBP12.6		
CyP C	23 kDa	retículo endoplásmico	FKBP13	13 kDa	retículo endoplásmico
CyP D	20 kDa	mitocondria	FKBP25 *	25 kDa	núcleo, citosol
CyP 40	40 kDa	citosol / receptor esteroides	FKBP38		
CyP NK			FKBP51	51 kDa	citosol / receptor progesterona
			FKBP52/59	52 kDa	citosol / receptor esteroides
			FKBP60		
			FKBP65		citosol

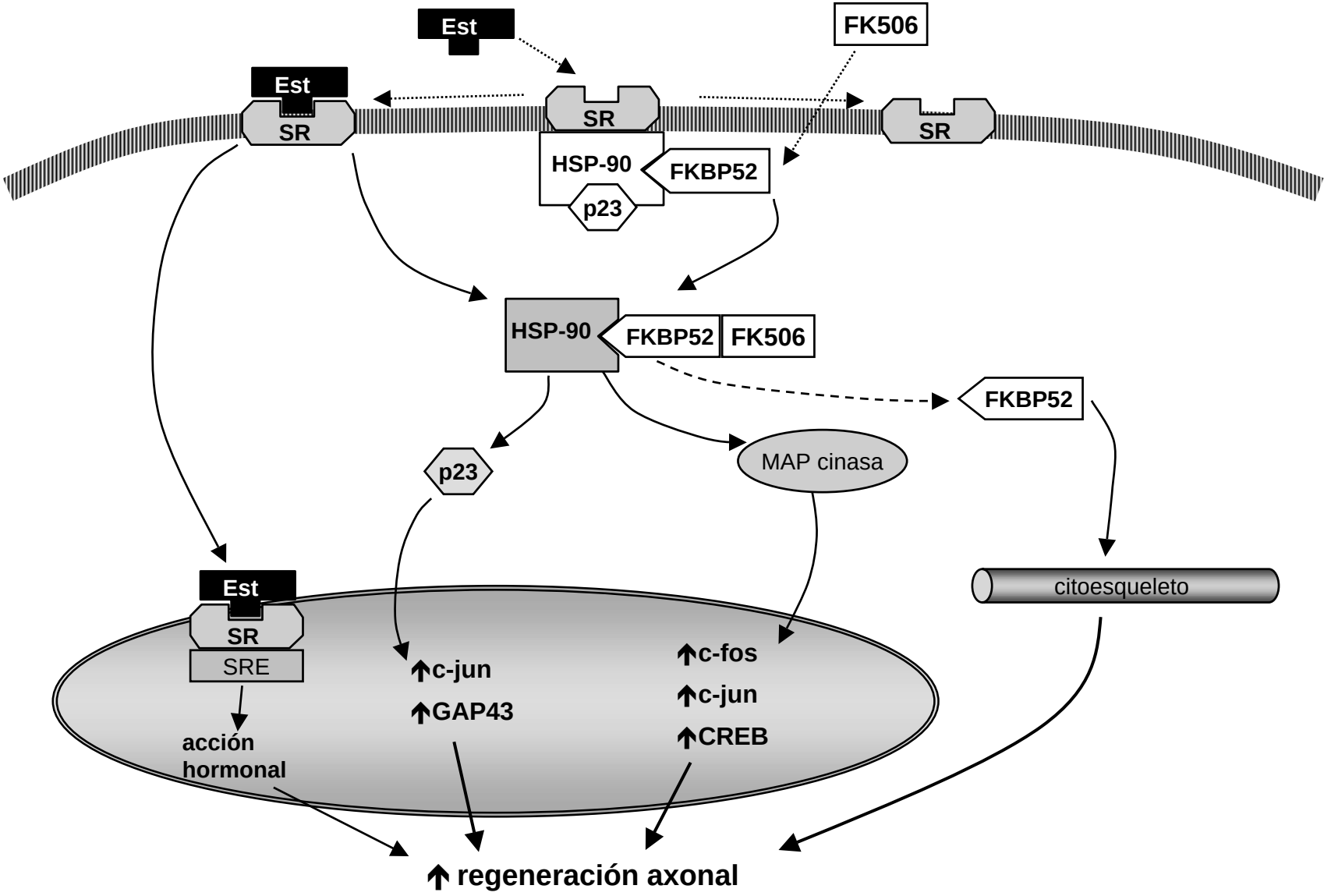
* baja afinidad por FK506 y alta afinidad por rapamicina

Tabla 2. Principales acciones biológicas inducidas por la unión de inmunosupresores a sus correspondientes inmunofilinas.

MECANISMO		EFECTO		FÁRMACO
Interacción con una inmunofilina asociada a algún complejo biológico	Disociación FKBP12-IP_3R	Desestabilización del canal iónico	Incremento de calcio intracelular	FK506 Rapamicina
	Disociación FKBP-RyR	Desestabilización del canal iónico	Incremento de tensión muscular	FK506 Rapamicina
	Interacción con FKBP52-receptor esteroide	Liberación de algún componente del complejo	Interacción con elementos del citoesqueleto	FK506
	Disociación FKBP12-TGFβR	Subunidad I queda libre	Activación del receptor	FK506
	Bloqueo asociación CyPD-MPTP	Inhibición MPTP	Mantenimiento del potencial de membrana mitocondrial	CsA
Formación de un complejo con la inmunofilina	Complejo FKBP12-FK506 Complejo CyP-CsA	Inhibición de la calcineurina	Bloqueo síntesis IL-2 en linfocitos T Inhibición NOS Bloqueo inactivación GAP43	FK506 CsA
	Complejo FKBP12-Rapamicina	Inhibición fosforilación proteína ribosomal	Bloqueo proliferación linfocitos T estimulada por IL-2	Rapamicina


antígeno
receptor T
Ca++
calmodulina
CsA
CyP
calcineurina
FK506
FKBP
NFAT
NFAT-P
transcripción
IL-2
IL-2
RAFT1
FKBP
Rapa
p70-S6
cinasa
P
p70-S6
cinasa
P
S6
mRNA
progresión del
ciclo celular


receptor NMDA
Ca++
Ca++
Ca++
Ca++
CsA
CyP
FK506
FKBP
calmodulina
calcineurina
BAD
P
BAD
nNOS
nNOS
P
arginina
citrulina
NO
ONOO-
MPTP
CsA
CyPD
BAD
bcl
activación de caspasas
muerte celular


Est
FK506
Est
SR
SR
SR
HSP-90
FKBP52
p23
HSP-90
FKBP52
FK506
FKBP52
p23
MAP cinasa
citoesqueleto
Est
SR
SRE
acción
hormonal
↑c-jun
↑GAP43
↑c-fos
↑c-jun
↑CREB
↑ regeneración axonal