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New strategy of tacrolimus administration in animal model based on tacrolimus-loaded microspheres

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ABSTRACT

New strategies for tacrolimus administration that conserve its immunosuppressive effect but avoiding fluctuations in tacrolimus circulating levels are needed. The aim was to analyze if subcutaneous biodegradable tacrolimus-loaded microspheres injection promoted a significant immunosuppressive response in rats. Rats received two subcutaneous tacrolimus-loaded microspheres injections at different days, the first injection was done at day 0 and the second injection was done 12 days after. Plasma circulating levels of tacrolimus, interleukin-2 (IL-2) and calcineurin phosphatase (PP2B) activity in mononuclear cells were measured. Tacrolimus plasma levels were significantly increased from the day after tacrolimus-loaded microspheres injection and remained increased during 10 days. Compared to control, plasma IL-2 levels and PP2B activity in mononuclear cells were significantly decreased during ten days. At day 12, a new subcutaneous injection of tacrolimus-loaded microspheres was performed and two days after injection, tacrolimus plasma levels were again increased and both IL-2 plasma levels and PP2B activity decreased. A single subcutaneous tacrolimus-loaded microspheres injection was enough to reduce tacrolimus-related immunosuppressive parameters. These results open the possibility of new therapeutic strategies to administrate calcineurin inhibitors reducing the variability of their circulating levels related to gastrointestinal drug absorption/metabolism modifications.

1. Introduction

Tacrolimus (FK506, Prograf) is a macrolide immunosuppressant agent indicated for the prophylaxis of organ rejection in patients receiving transplantation [1]. Tacrolimus bonds to an immunophilin, FK506 binding protein (FKBP12), forming a complex that inhibits calcineurin phosphatase. Calcineurin phosphatase dephosphorylates the nuclear factors of activated T cells (NFAT), thereby inducing their translocation to the cell nucleus, essential processes for activating cytokine gene expression and, consequently, the immune response [2].

Tacrolimus administration has been associated with severe side effects, including hypertension, nephrotoxicity, and diabetes, that may compromise not only renal graft but also the patients survival [3–5]. Indeed, tacrolimus nephrotoxicity occurs in 17% to 44% of renal transplant recipients and in 18% to 42% of liver transplant recipients [6,7].

Despite of the well-established immunosuppressive effects of tacrolimus, its therapeutic use is complicated due to its narrow therapeutic window index [8–11]. Moreover, tacrolimus shows considerable inter-intra-patient variability in pharmacokinetics profile and a poor oral bioavailability related to its poor solubility [12,13]. Therefore, guide dose modifications continuous monitoring of circulating levels of tacrolimus is required. In this regard, conventional chronic tacrolimus treatment is based on one or two intakes/daily, as well as an alternative intravenous route indicated for treatment early after transplantation or to cases in which oral route is unavailable. Accordingly, large number of studies has supported that in many patients tacrolimus treatment is subjected to fluctuations in the levels of circulating tacrolimus [8,10]. The existence of such fluctuations has been attributed to many factors including non-adherence to treatment but also by variability in drug absorption/metabolism [14–16]. Therefore, new strategies for tacrolimus therapy

Abbreviations: FKBP12, FK506 binding protein; IL-2, interleukin-2; PP2B, calcineurin phosphatase; PLGA, poly-D,L-lactic-*co*-glycolic acid.

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are obviously needed to obtain prolonged blood tacrolimus concentrations preserving its immunosuppressive effects but avoiding tacrolimus peaks that promote under- and over-tacrolimus dosing.

Biodegradable polymers poly-D,L-lactic-*co*-glycolic (PLGA) acid has been widely used to form microspheres with capacity to encapsulate drugs allowing continuous long-term delivery of them [17]. The well recognized low toxicity of the polyesters and their degradation products make possible its utilization as a biodegradable drug delivery system without side effects [18,19]. This microspheres system has been successfully used in treatments of different diseases, avoiding the inconvenient and limitations related to oral chronic treatments [20,21].

Taken together, the aim of the present study was to analyze if a subcutaneous injection of tacrolimus-loaded microspheres in rats may promote significant immunosuppressive response.

2. Material and methods

2.1. Experimental design

Experiments were completed in male Wistar Kyoto with age range between 12 and 14 weeks with similar body weight (150–200 g). Rats were maintained in temperature-controlled room under 12-hour dark/light cycles and free access to food (standard laboratory chow) and water. All rats received a subcutaneous injection of tacrolimus loaded microspheres that were prepared just before the injection (11.4 mg of tacrolimus loaded microspheres were dispersed in 2 mL of saline solution). Animals were not fasted before administration. Rats were anesthetized by injection of sodium pentobarbital (50 mg/kg) and exsanguinated recollecting the blood in EDTA-containing tubes. Fig. 1 shows the experimental design schedule in which was used 48 rats (six rats for each experiment time).

All animal care, use and experimental protocols were approved by the Ethics Committee of Complutense University, responsible of the correct application of the order 86/609/CEE (Spanish order 1201/2005).

2.2. Preparation of tacrolimus-loaded microspheres

Preparation of microspheres was performed by a spray-drying process (Mini Spray-Dryer B-191, Büchi, Flawil, Switzerland). To obtain microspheres containing tacrolimus, both PLGA 50/50 (1.8 wt%) and tacrolimus (Sigma, 109581-93-3) (0.2 wt%) were dissolved in dichloromethane and mixed. The polymeric solution (100 mL) was maintained under constant stirring (900 rpm) for 30 min and sprayed through the nozzle of the spraydryer. Assay conditions were: inlet air temperature 60 °C, outlet air temperature 36–40 °C, spray flow (represented as the volume of the air input) 800NL/h, pump: 16%, aspirator: 70%. Microspheres were collected from the spray-dryer cyclone and stored under dry conditions.

Before being subcutaneously injected in the rats, the efficiency of entrapment of tacrolimus in the microspheres was estimated. In this regard, it is known that both PLGA and tacrolimus are soluble in chloroform. A maximum absorbance at 300 nm has been shown after UV/V spectra (210–450 nm) of the drug in chloroform whereas the polymer solution did not absorb at this wavelength. Therefore, to determine the amount of tacrolimus included in the polymeric microspheres, 10 mg of the drug-loaded microspheres were dissolved in 1 mL of chloroform. The absorbance at 300 nm was interpolated in a standard curve of tacrolimus soluted in chloroform in the range of 0.16–2.5 mg/mL. The amount of drug entrapped in the microspheres was then calculated as drug loading = weight of tacrolimus in microspheres/microsphere sample weight. The result was expressed as percentage of entrapment efficiency with respect to the total amount of tacrolimus added to microspheres mixture. The reached efficiency of entrapment of tacrolimus in the microspheres was approximately 42.5%.

2.3. Determination of tacrolimus and interleukin-2 (IL-2) plasma levels

Tacrolimus and interleukin-2 (IL-2) plasma levels were both measured by enzyme-linked immunoabsorbent assays (ELISA) kits. ELISA kits were purchased from Diasorin Laboratory (PRO-Trac™ II Tacrolimus ELISA kit) for tacrolimus and from Invitrogen (Rat Interleukin-2, KRC0021) for IL-2 and they were performed following the manufacturer instructions. The FK506 ELISA kit has as sensitivity 0.27 ng/mL with and intra-assay variation ranging between 6.5%–9.8%. The IL-2 ELISA kit showed as sensitivity 5 pg/mL with an intra-assay coefficient variation ranging between 5.5%–7.5%.

2.4. Measurement of calcineurin phosphatase activity in mononuclear cells

As previously we reported [22], mononuclear cells were isolated using Ficoll-Hypaque and resuspended in RPMI-1640 medium supplemented with 5 mmol/L L-glutamine and 0.25% serum albumin. Mononuclear cells were obtained and manipulated under sterile conditions. To obtain enough amounts of mononuclear cells for each experimental time, blood from each two rats was pooled. Therefore, Calcineurin phosphatase activity was determined in three different samples of blood from 6 rats coupled in pairs. In the isolated mononuclear cells, calcineurin phosphatase activity was measured using a colorimetric assay kit (Enzo Life Science, BML-AK816), based in the releasing of free-phosphate from the calcineurin phosphate substrate RII phosphopeptide by the Malachite green assay [23]. The ELISA kit was performed following the manufacturer's instructions at least in three rats in each experimental time.

2.5. Statistical analysis

The results were represented as mean ± SEM. To determine the statistical significance, Mann-Whitney test was used by software SPSS 15.0. p value < 0.05 was considered statistically significant.

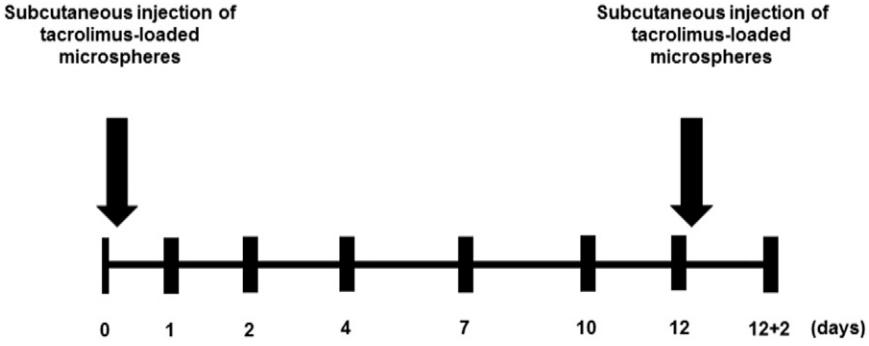

Fig. 1. Experimental design Blood samples were collected at 0, 1, 2, 4, 7, 10, 12 days after a single subcutaneous injection of tacrolimus-loaded microspheres. Twelve days after this first subcutaneous injection, a second subcutaneous injection of tacrolimus-loaded microspheres was done and two days after blood samples were collected again.