Transpl Int (2005) 17: 841–847
DOI 10.1007/s00147-004-0790-8

ORIGINAL ARTICLE

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Drug delivery system using microspheres that contain tacrolimus in porcine small bowel transplantation

Received: 19 February 2004
Revised: 14 July 2004
Accepted: 9 September 2004
Published online: 25 May 2005

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Abstract Rejection remains a major barrier to successful bowel transplantation, in spite of improved immunosuppressive techniques. Therefore, new, more effective, immunosuppressants, with fewer side effects, are needed. Biodegradable microspheres containing tacrolimus (FK506) were used in an experimental porcine small bowel transplantation. Twenty pigs underwent transplantation and were divided into four groups according to the immunosuppressive regimen. Group A ($n=5$): no immunosuppression; group B ($n=6$): 0.2 mg/kg per day of FK506; group C ($n=3$): 1.0 mg/kg per day of FK506; group D ($n=6$): 0.04 mg/kg per day of FK506 contained in biodegradable microspheres. Rejection was diagnosed macroscopically by endoscopic examination and histologically by biopsy specimen analysis. The mean survival time and standard deviation (SD) were 8.8 ± 3.5, 11.0 ± 1.4, 9.7 ± 2.5 and 28.6 ± 22.5 days for groups A, B, C, and D, respectively, with a statistically significant difference found between group D, on the one hand, and groups A, B and C, on the other. The mean trough blood concentration of FK506 was 10.5 ± 2.2, 27.9 ± 6.0 and 10.5 ± 3.5 ng/ml in groups B, C and D, respectively. In groups A and B, all pigs died of rejection, without infection. In group C, all died of infection, without rejection. In contrast, none of the pigs in group D developed rejection or infection. Our results clearly show that the drug delivery system using biodegradable microspheres that contain FK506 is effective for controlling rejection with fewer side effects in the porcine small bowel transplantation.

Keywords Tacrolimus · Small bowel transplantation · Microsphere · Drug delivery system

Introduction

Despite improvements in immunosuppressive therapy over the past 10 years (e.g., FK506), small bowel transplantation has not been widely used as an alternative to long-term intravenous nutritional support for patients with intestinal failure. This is largely because the bowel has a large lymphoid component and unique immunopathology, which causes rejection. This means that new, more effective, immunosuppressants, with fewer side effects, are needed. Recently, many new drug delivery systems (DDSs) have been developed, and the utility of biodegradable microspheres has been reported in several papers [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18]. Microspheres of certain sizes were mainly taken up by Peyer's patch, where they succumbed to phagocytosis by macrophages and released their contents continuously for 80 days [19, 20, 21, 22, 23, 24, 25]. Therefore, oral administration of microspheres containing FK506 may be more effective for the

prevention of rejection in small bowel transplantation and may have fewer systemic side effects than FK506 alone. That is because local tissue concentrations of FK506, in the case of FK506 microspheres, may be higher than those in blood and, thus, may control immune-regulating cells more effectively in the graft's mucosa. In our study, we used a new type of microsphere containing FK506 for an experimental porcine small bowel transplantation and determined whether they can prevent small bowel allograft rejection.

Materials and methods

Preparation of microspheres containing FK506

PDLLA was synthesized by simple polycondensation of D,L-lactic acid at 180°C under reduced pressure without any catalyst. The average molecular weight was 9,000, as determined by gel permeation chromatography, relative to polystyrene standard samples. Poly-D,L-lactic acid (PDLLA) microspheres containing FK506 were prepared by the solvent evaporation method using double emulsion. In brief, 40 mg of FK506 aqueous solution was poured into 1 ml of methylene chloride, containing 200 mg of PDLLA, followed by emulsification by probe sonication, to form an emulsion. The emulsion was added into 2 ml of 1% polyvinyl alcohol aqueous solution, which had been saturated with methylene chloride at room temperature and agitated by a vortex mixer to form a double emulsion. The double emulsion was stirred by an impeller (200 rpm) at room temperature until the methylene chloride was completely evaporated. The microspheres were collected by centrifugation (5,000 rpm, 5 min, 4°C), washed three times with cold distilled water by centrifugation, and finally lyophilized. The size of the prepared microspheres was assessed from light microscopic photographs according to a reference scale, and their diameters were confirmed to be 7–10μm, which is an appropriate size for accumulation at Peyer's patch.

Distribution of PDLLA microspheres on the small bowel of the pigs

A pig was orally administered PDLLA microspheres containing coumarin 6 and was killed 24 h later, and a part of the jejunum was removed for examination. The excised tissues were mounted in OTC freezing compound and frozen in liquid nitrogen. They were cut into 4–6μm serial sections, which we viewed on a fluorescence microscope to confirm the distribution of PDLLA microspheres on the small bowel.

Model for local tissue and blood concentrations of FK506

Four pigs underwent tube gastrostomy and were divided into two groups according to their immunosuppressive regimen: group 1, 0.2 mg/kg per day of FK506 and group 2, 0.04 mg/kg per day of FK506 in microspheres. Each of the experimental groups consisted of two pigs. Pigs were killed on the 6th day, and part of the jejunum was removed for examination. Blood concentrations of FK506 were monitored at 1 h, 2 h, 4 h, 8 h, and 12 h, and, on the 6th day after the start of administration, FK506 blood levels were determined by a microparticle enzyme immunoassay (Abbott IMX) and tissue concentrations were measured by high-performance liquid chromatography/tandem mass spectrometry.

Transplantation model

Outbred, unrelated pigs were used in this study. Donors were Camborough pigs with a mean and SD weight of 16.8 ± 2.6 kg, and recipients were Hypor pigs with a mean and SD weight of 23.0 ± 2.6 kg. Allogenicity between donors and recipients was confirmed by the mixed lymphocyte reaction. All animal experiments were carried out according to the principles of laboratory animal care [26]. The porcine models were divided into four groups according to immunosuppressive regimen: group A, no immunosuppression; group B, 0.2 mg/kg per day of FK506; group C, 1.0 mg/kg per day of FK506; group D, 0.04 mg/kg per day of FK506 contained in biodegradable microspheres administered orally.

Food was withheld for 24 h before surgery for both of donors and recipients, and no bowel decontamination was performed.

For pre-medication, atropine sulfate (13 μg/kg, intramuscularly) and ketamine hydrochloride (20 mg/kg, intramuscularly) were used.

Blood access was achieved by cannulation to the ear vein, which was used for the administration of pentobarbiturate (10 mg/kg). After intubation, general anesthesia was maintained with GOF (halothane 0.5%–1.0%) and muscle relaxants. In the recipients, arterial blood pressure was monitored with a line into the internal jugular artery. Access to the external jugular vein was obtained for intraoperative and postoperative transfusion as well as for the taking of blood samples.

The pigs that died within 6 days were omitted from this study as technical failures.

Donor operations

Through a midline incision, the superior mesenteric artery (SMA) was identified at its takeoff from the

aorta. The portal vein (PV) was dissected free from the uncinate process to the level of the hepatic hilum. The splenic vein and all pancreatoduodenal vessels were ligated and divided. The small intestine was cut at the jejunum (15 cm distal to Treitz's ligament) and at the terminal ileum, with its lumen closed by ligation. Once the small intestine was dissected completely free and with only its vascular pedicle attached, a cannula was inserted into the infrarenal aorta. After 2,000 units of heparin had been administered for systemic heparinization, the aorta was ligated proximal to the takeoff of the celiac trunk. After 20 mol of potassium chloride had been infused into the heart to kill the pig, the small bowel was flushed with 500 ml of lactated Ringer's solution (RL) at 4°C. The SMA was preserved with a cuff of the aorta (the distal end was closed with a running suture of 5–0 Prolene). The PV was cut at the hepatic hilum. The graft was placed in a basin containing RL at 4°C. The intestinal lumen was not flushed.

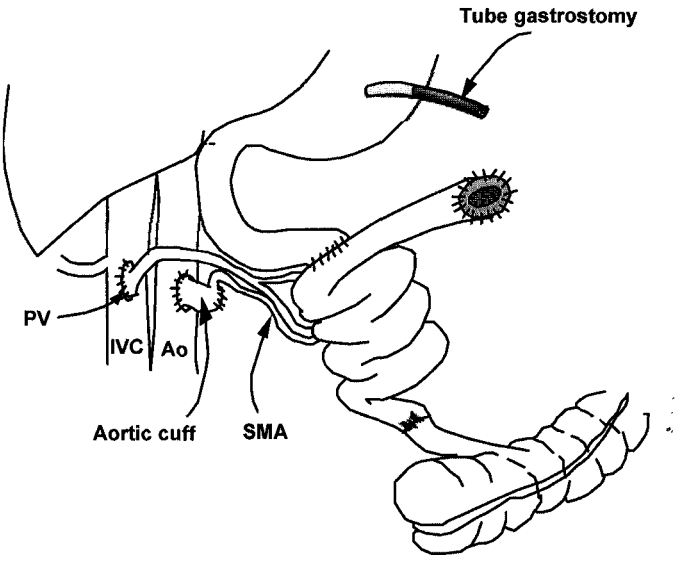

Fig. 1 Schema of recipient's operation (*SMA* superior mesenteric artery, *PV* portal vein, *Ao* aorta, *IVC* inferior vena cava)

Recipient operations

Through a midline incision the small bowel was resected from the jejunum (15 cm distal from Treitz's ligament) to the terminal ileum. At the same time all lymph channels were ligated and cut to prevent postoperative lymphorrhea, and all branches of the SMA were ligated and divided at the takeoff except the colonic arteries. The aorta and inferior vena cava were prepared for engraftment, while all para-aortic lymph channels were ligated and divided to prevent lymphorrea after transplantation. The graft was flushed again with 500 ml of RL and then placed into the recipient. After administration of 2,000 units of heparin, the aorta was clamped longitudinally, and the donor's SMA, with the aortic cuff, was anastomosed to the recipient's infrarenal aorta in the end-to-side fashion with a running 5–0 Prolene suture. The donor's PV was then anastomosed to the recipient's infrarenal inferior vena cava (IVC) in the end-to-side fashion with a running 5–0 Prolene suture. After revascularization of the graft, the pigs received 20 ml of 8.4% sodium bicarbonate, 0.5 mol of calcium chloride and 20 ml of mannitol. Jejunostomy was performed at the proximal end of the graft for postoperative endoscopic examination. The recipient's jejunum was anastomosed to the graft's jejunum in the end-to-side fashion by the two-layer technique. The recipient's terminal ileum was anastomosed to the graft's ileum, end-to-end, by the two-layer technique. Tube gastrostomy was performed for the administration of FK506 or FK506 microspheres with an 8 Fr. feeding tube (Fig. 1).

Postoperative care

For the first 3 days the pigs received 500 ml of RL with 5% dextran, 0.5 g cefazolin sodium, 20 mg furosemide and 0.2 mg buprenorphine hydrochloride every day. They had access to water on postoperative day (POD) 1 and resumed a normal diet on POD 4, after which blood access was removed. Immunosuppressive drugs were administered daily through the gastrostomy tube according to the above-mentioned regimen. Those losing more than 30% of their initial body weight were killed according to the protocol guidelines set by the University of Minnesota's Research Animal Resource Committee.

Postoperative monitoring

Endoscopic examination, biopsy sampling of intestinal mucosa, blood sampling, and body weight measurement were performed twice a week (on days 7, 10, 14, 17, 21 and so on). Rejection was diagnosed macroscopically with endoscopy and histologically by biopsy specimen analysis.

Histology

Tissue samples for histological examination were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and examined by hematoxylin–eosin staining. The diagnosis and grading of rejection were defined according to the previous report [27] (Table 1).

Table 1 Grading system for small bowel allograft rejection

Rejection grade	Histological findings
None	
Mild	Mild inflammation of the lamina propria, which may extend into the submucosa; minimal individual cell necrosis; mild villous blunting
Moderate	Inflammation of the lamina propria and submucosa, which may extend into muscularis propria; easily identifiable cell necrosis; varying degree of villous blunting
Severe	Inflammation of the lamina propria, submucosa and muscularis propria. Diffuse individual epithelial cell necrosis with possible loss of contiguous portions of the epithelium; complete villous blunting

Statistics

Pig survival rates were calculated by the Kaplan–Meier method. We used the log-rank test to compare differences in the pig survival rates and FK506 trough levels between the four groups. Values of $P < 0.05$ were considered to be statistically significant.

Results

Distribution of PDLLA microspheres on the small bowel of the pigs

We confirmed that PDLLA microspheres were taken up by only Peyer's patch on the porcine model.

Local tissue and blood concentrations of FK506

The mean ± SD tissue concentrations in the jejunum were 18.2 ± 11.6 ng/ml and 1.6 ± 0.08 ng/ml for the 0.2 mg/kg per day and 0.04 mg/kg per day microsphere groups, respectively, while blood concentrations on the same day were 8.1 ± 2.7 ng/ml and 6.7 ± 4.1 ng/ml, respectively.

Pharmacokinetics of microspheres containing FK506

The pharmacokinetics of microspheres are shown in Fig. 2. Blood FK506 concentrations of the group administered FK506 alone peaked at 2 h after administration, while those of the group administered FK506 in microspheres did not peak.

Survival

Six pigs died within 6 days postoperatively. They were excluded from this study as technical failure. The mean ± SD survival period was 8.8 ± 3.5, 11.0 ± 1.4, 9.7 ± 2.5 and 28.6 ± 22.5 days for groups A ($n = 5$), B ($n = 6$), C ($n = 3$), and D ($n = 6$), respectively (Fig. 3). All animals in groups A, B and C died within 15 days, but two of five animals in group D survived more than 45 days, with a statistically significant difference between group D and groups A, B and C.

Causes of death

Causes of death are shown in Table 2. In group A, three pigs died of rejection. Another two died of internal hernia and arterial thrombosis, but both of them also suffered from rejection. In group B, all of six pigs died of rejection, without infection. In group C, all of three pigs died of sepsis, without rejection. One pig suffered from intraperitoneal abscess, pneumonia, pericardial abscess and pyelonephritis, and the others suffered from intraperitoneal abscess and pneumonia. In contrast, none of the six pigs in group D developed either rejection or infection. The causes of death in group D were loss of massive ascites, leakage of intestinal anastomosis,

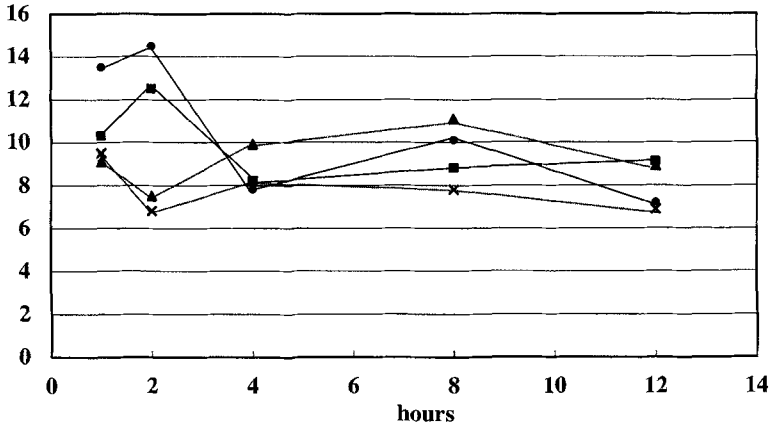

Fig. 2 Pharmacokinetics of microspheres (*circles* FK1, *squares* FK2, *crosses* FKMS1, *triangles* FKMS2)

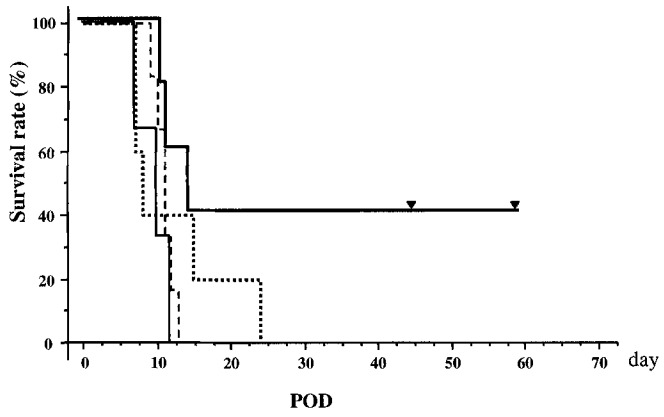

Fig. 3 Survival time of the pigs (*dashed lines* group A, *dotted lines* group B, *narrow solid line* group C, *broad solid line* group D, *triangles* pigs killed)

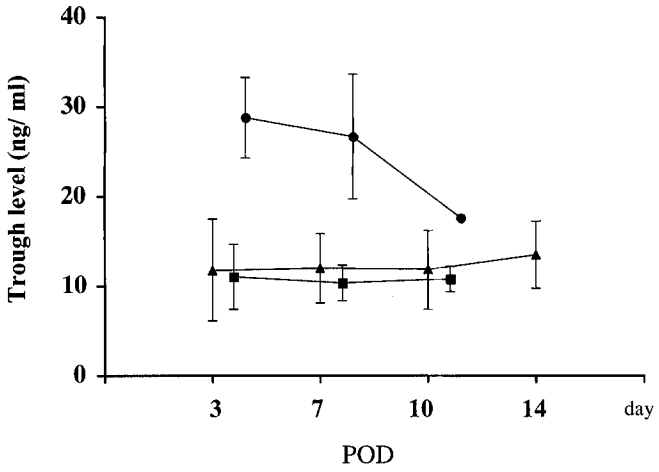

Fig. 4 Post-transplantation 24 h trough levels of FK506 (*circles* group B, *squares* group C, *triangles* group D)

partial necrosis of small intestine and being killed because of weight loss.

Changes in blood concentration of FK506

Post-transplantation 24 h trough levels of FK506 are shown in Fig. 4. The mean ± SD 24 h trough level on POD 3 was11.3±3.7, 28.7±4.5, and 11.7±5.7 ng/ml for groups B, C, and D, respectively, while the corresponding values on POD 7 were 10.3±2.0, 26.7±6.9 and 11.9±3.9 ng/ml. Blood concentrations for group C were statistically higher than those for the other two groups on both days. There was no statistically significant difference between groups B and D.

Degrees of rejection

Three of five pigs in group A and three of six in group B showed severe rejection on POD 7, and, eventually, all the pigs in both groups showed severe rejection. In contrast, none of the animals in groups C and D showed rejection at any time (Table 3).

Table 2 Cause of death

Group	Cause of death	Number
Group A ($n=5$)	Internal hernia	1
	Arterial thrombosis	1
	Rejection	3
Group B ($n=6$)	Rejection	6
Group C ($n=3$)	Sepsis [intraperitoneal abscess, pneumonia, pericardial abscess, pyelonephritis ($n=1$) intraperitoneal abscess, pneumonia ($n=2$)]	3
Group D ($n=5$)	Massive ascites	1
	Leakage of intestinal anastomosis	1
	Partial necrosis of small intestine	1
	Killed	2

Discussion

Recently, many new DDS have been developed, and the usefulness of biodegradable microspheres has been reported in many papers. For example, Nakase et al. reported that oral administration of dexamethasone (DX) microspheres ameliorates mucosal injury in 2,4,6-trinitro-benzene sulfonic acid-induced colitis more effectively than DX alone and suggested that this DDS can be used for inflammatory bowel diseases [28]. They also reported that DX can be released continuously from DX microspheres and that these microspheres are mainly taken up by the inflamed colon. Tabata et al. reported that microspheres of some size (7 μm–10 μm) were mainly absorbed by Peyer's patch, where they were underwent phagocytosis by macrophages and were able to release their contents continuously for 80 days [29, 30, 31, 32].

Similarly, we tried to examine more potent inhibitory effects of a new type of microsphere containing FK506 in experimental porcine small bowel transplantations

Table 3 Degrees of rejection

Group	Rejection	Day 7	Day 10	Day 14	Day 17
Group A ($n=5$)	None	2	1		
	Moderate		1		
	Severe	3		1	
Group B ($n=6$)	None	2			
	Moderate	1	3		
	Severe	3	3		
Group C ($n=3$)	None	3	2		
Group D ($n=5$)	None	5	5	3	2

than could be attained with free FK506. We confirmed that the PDLLA microspheres were taken up only by Peyer's patch in the porcine model, which is in agreement with previous reports suggesting that intestinal Peyer's patches are the major sites of orally administered particulate uptake in mice, rats, rabbits and calves. We then attempted to confirm whether local tissue concentrations of FK506 were higher in the case of FK506 microspheres than with FK506 alone, but these results were in contrast to what was expected. Further investigation regarding the absorption of FK506 is required to account for this observation.

In addition, we confirmed that PDLLA microspheres continuously released their contents. Therefore, microspheres containing FK506 may more effectively control immune-regulating cells in the graft mucosa, and oral administration may be more effective in preventing rejection of small bowel transplantation and may have fewer side effects than administration of FK506 alone.

The difference in the survival time between group D and groups A, B, and C is statistically significant. Additionally, 40% of the pigs in group D (two of five) survived more than 45 days, while all other pigs died within 14 days. Moreover, all the pigs in group D showed neither rejection nor infection, while in groups A and B all pigs developed rejection, and in group C all pigs died of infection.

At the same time, there was no statistically significant difference in the mean 24 h blood trough level of FK506 between group D and group B, while that in group C was statistically higher. The blood trough level of group D was similar to that in group B, even though the quantity of FK506 administered to group B was five times that in group D. Previous studies have reported that the bioavailability of FK506 is dependent on the intestinal surface area and transit time [33]. It is now acknowledged that active secretion by P-glycoprotein, a membrane efflux pump, from enterocyte into lumen, as well as intestinal metabolism by cytochrome P450IIIA4, plays an important roles [34]. This has led us to speculate that these findings may also account for the fact that FK506 microspheres were not drawn out from the wall of the graft by P-glycoprotein and were not metabolized in the small intestine during its absorption by cytochrome P450IIIA4, as was the case with FK506 alone.

In the case of FK506 microspheres, local tissue concentrations of FK506 were lower than when FK506 alone was administered, although pigs administered FK506 microspheres survived longer. This may be because the PDLLA microspheres released their contents continuously at Peyer's patch, where they suffered phagocytosis by macrophages, and, thus, more effectively controlled immune-regulating cells in the graft mucosa.

In conclusion, if the FK506 trough levels are similar, pigs administered FK506 microspheres survive longer and have a lower rate of infection than those administered FK506 alone. Our results show that a drug delivery system using microspheres containing FK506 is more effective for controlling rejection and has fewer side effects in experimental small bowel transplantation.

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