Pharmacokinetics of Intra-arterial Delivery of Tacrolimus to Vascularly Isolated Rabbit Forelimb

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ABSTRACT

A vascularly isolated rabbit forelimb model simulating conditions of composite tissue allografting was used to determine the regional pharmacokinetic advantage achievable in extremity tissue components during i.a. tacrolimus (FK506) administration. FK506 was infused continuously via osmotic minipump into the right brachial artery of New Zealand rabbits at 0.05, 0.1, and 0.2 mg/kg/day. On day 6, FK506 concentrations were measured in aortic whole blood, heart, lung, liver, kidney, spleen, and fat, as well as in skin, muscle, bone, and bone marrow samples from both right and left forelimbs. The relative tissue concentrations of FK506 in descending order were [spleen ≈ lung ≈ kidney] > [heart ≈ skin ≈ muscle] > [fat ≈ bone marrow] > [liver ≈ bone ≈ blood]. In marked contrast to previous results with i.a. cyclosporin A infusion, only a minimal regional advantage of local FK506 delivery (mean right/left concentration ratios 1.0–1.4) was obtained in all forearm tissues over the dose range studied. For each limb tissue, left-sided FK506 concentrations significantly correlated with systemic blood levels, and the left-sided tissue-to-whole-blood concentration ratio did not vary significantly with dose. We conclude that FK506 is pharmacokinetically inferior to cyclosporin A for continuous i.a. administration to the vascularly isolated rabbit forelimb, and hypothesize that this difference is the result of differences in the distribution of each drug within whole blood. Our findings suggest that, despite its demonstrated efficacy in experimental and clinical transplantation, FK506 would not be an appropriate immunosuppressant to deliver via the i.a. route for prevention of limb allograft rejection.

The single most important obstacle currently preventing limb transplantation from becoming a clinical reality is not the inability to technically perform the surgical procedure or to restore function, but rather the lack of specific, safe, and effective immunosuppressive therapy. Studies of hand, partial hand, and neurovascular free flap transplantation in nonhuman primates have stressed the need for using continuous, high-dose, maintenance immunosuppression to ensure long-term allograft survival (Skanes et al., 1986; Stark et al., 1987; Stevens et al., 1991). However, even under these conditions, rejection episodes still occurred, requiring high-dose steroid or monoclonal antibody treatment, with subsequent development of significant drug-induced toxicity. Although chronic systemic administration of relatively high doses of nonspecific immunosuppressive agents is readily accepted in the visceral organ transplant recipient faced with poor quality of life or death, this would at present be unacceptable in the patient requiring musculoskeletal reconstruction (Paskert et al., 1987).

One approach toward reducing the drug-specific and general adverse consequences of systemic immunosuppression in recipients of extremity composite tissue allografts, and thereby improving the clinical feasibility of the limb transplant procedure, is the use of local drug administration systems (Gruber, 1992). During the past decade, favorable experiences with pump-based local immunosuppressive therapy have been reported in multiple rat and canine visceral solid-organ allograft models by our laboratory (Gruber et al., 1989, 1992) and others (Ruer et al., 1986; Stepkowski et al., 1989; Kamei et al., 1991; Ko et al., 1994). It is our hypothesis, given the relatively low blood flow to the limb, that direct (i.a.) extremity infusion of appropriately chosen nonspecific immunosuppressants will greatly increase local tissue drug levels when compared with same-dose i.v. treatment, thereby improving the therapeutic index and allowing...

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ABBREVIATIONS: Cl_s, systemic clearance; CSA, cyclosporin A; FK506, tacrolimus; K_d, equilibrium distribution ratio; Q_T, target organ blood flow; RBC, red blood cell.
for rejection prophylaxis with decreased systemic drug exposure. Along these lines, we developed a novel rabbit forearm model to investigate the pharmacokinetic parameters of continuous i.a. pump-based drug delivery to the extremity while simulating the conditions existing following composite tissue allografting (Shirbacheh et al., 1999b). In previous studies, we demonstrated that with optimal dosing, a large (4- to 14-fold) regional pharmacokinetic advantage could be achieved in locally treated skin, muscle, bone, and bone marrow during i.a. cyclosporin A (CSA) infusion when compared with tissues in the contralateral limb in the presence of low systemic blood levels (Shirbacheh et al., 1999a).

Over the past several years, tacrolimus (FK506) has emerged as one of the major alternative immunosuppressant to CSA around which multidrug antirejection regimens for clinical solid-organ transplantation have been based (The U.S. Multicenter Liver Study Group, 1994; Pirsch et al., 1997; Gruessner, 1997). Moreover, in rodent models, FK506 therapy has been recently demonstrated to significantly prolong vascularized hindlimb allograft survival (Fealy et al., 1994, 1995); suppress rejection of skin allografts with minimal systemic drug levels when applied as a topical ointment (Yuzawa et al., 1996; Fujita et al., 1997); permit normal regeneration of sciatic nerve allografts (Buttemeyer et al., 1995); and significantly increase the rate of sciatic nerve axonal regeneration after crush injury (Wang et al., 1997).

Based on the above favorable clinical and experimental data, FK506 clearly has enormous potential for use in preventing rejection of composite tissue transplants, and the pharmacokinetics of its local delivery are worthy of study in our vascularly isolated rabbit forearm model. Therefore, the primary goals of the present study were to determine 1) tissue drug concentrations and concentration ratios in both locally treated and systemically treated (contralateral) limbs, 2) the regional advantage attainable in various limb tissues in relation to systemic blood levels, 3) the generalized tissue distribution of drug, and 4) the equilibrium distribution ratio for systemically treated tissues during i.a. FK506 infusion at clinically relevant doses.

Materials and Methods

Animals. Eighteen outbred male New Zealand rabbits, 2.8 to 3.0 kg in weight, were used in our studies and cared for in accordance with guidelines established by the Institutional Animal Care and Use Committee of the University of Louisiana School of Medicine. Rabbits were housed in separate cages at constant room temperature with a 12-h light/dark cycle and maintained on a balanced rodent diet with free access to water throughout the experiment.

Rabbit Model. Alzet 2 ML1 miniosmotic pumps (Alza Corporation, Palo Alto, CA) were used for continuous i.a. drug administration in our rabbit forearm model of local immunosuppression as described in detail previously (Shirbacheh et al., 1999b). In brief, the distal end of a composite Intramedic PE-50/PE-10 infusion catheter (Clay Adams, Parsippany, NJ) was inserted into the right brachial artery via the thoracodorsal artery branch. The pump attached to the other end of the catheter was placed in a s.c. pocket overlying the serratus anterior muscle. Ligation of all muscles at the right midarm level was performed to eliminate collateral circulation and simulate allografting. Pumps were filled with FK506 solution (Prograf, 5 mg/ml; Fujisawa USA, Inc., Deerfield, IL) and diluted with dehydrated alcohol to achieve final concentrations of 2.5, 1.25, and 0.625 mg/ml.

Pharmacokinetic Study. FK506 was administered by continuous i.a. infusion at 0.05 (n = 6), 0.10 (n = 6), and 0.20 (n = 6) mg/kg/day. On postoperative day 6, animals were anesthetized with an i.m. injection of ketamine (37.5 mg/kg) and xylazine (5 mg/kg). Following left thoracotomy, a blood sample was drawn from the aorta for determination of whole-blood FK506 concentration, the aorta was transected, and the animal euthanized by bleeding. The heart, lower lobe of the right lung, right lobe of the liver, right kidney, spleen, and retroperitoneal fat were removed from all animals for determination of FK506 concentration. Skin, muscle, bone, and bone marrow tissue samples were obtained symmetrically from both right and left forelimbs for determination of FK506 levels. All samples were stored at −80°C until analysis.

FK506 Assay. Whole-blood FK506 concentrations were measured using the INCSTAR ProTrac II enzyme-linked immunnoassay method (MacFarlane et al., 1996). Tissue specimens were extracted into methanol before analysis. Each sample was weighed, placed in a labeled polypropylene tube, and ice-cold methanol (reagent grade) added to give 40 mg tissue/ml methanol. The extraction tubes were placed in an ice bath and the contents homogenized using the PowGen Homogenizer (Fischer Scientific, Pittsburgh, PA) at 25,000 rpm for 10 to 15 s until the tissue was totally fragmented. Each tube was capped and frozen at −70°C until analysis. Just before analysis, each extract was thawed, well mixed, transferred to a 2-ml microtube, and centrifuged at 10,000g for 4 min. Reacti-Therm III dry-block (Pierce, Rockford, IL) was used to evaporate duplicate 100-μl aliquots of supernatant. After reconstituting FK506 in diluted conjugate (horsearadish peroxidase) solution, duplicate 200-μl aliquots were transferred to a microtitr plate. Anti-FK506 monoclonal antibody was then added to each well and the remainder of the assay was performed according to the original ProTrac II enzyme-linked immunoassay whole-blood method. Controls were assayed with each batch to determine run acceptability. Tissue extracts with FK506 concentrations greater than 30 ng/ml were diluted appropriately with methanol until they were within the assay range. Values were expressed as ng FK506/g tissue. Measured FK506 concentrations in nonlimb tissues were corrected for residual blood content as described previously for CSA (Bernareggi and Rowland, 1991; Shirbacheh et al., 1999b).

Data Analysis. Right (locally treated) and left (systemically treated) forearm mean tissue FK506 levels, as well as mean skin/muscle, skin/bone, and skin/bone marrow FK506 concentration ratios, were compared at each dose level using a paired Student’s t test. The regional pharmacokinetic advantage of i.a. FK506 infusion for each tissue at each dose was determined from the right/left (R/L) ratio of tissue FK506 concentrations. The equilibrium distribution ratio (Kd) at each dose was calculated for limb tissues from the left-sided tissue-to-whole-blood FK506 concentration ratio and for nonlimb tissues from the tissue-to-whole-blood drug concentration ratio (Bernareggi and Rowland, 1991). A one-way ANOVA was used to determine whether right- and left-sided tissue FK506 concentrations and concentration ratios and Kd values were related to local FK506 dose. The Scheffe’s F procedure was used for post hoc, multiple, paired comparisons. The linearity of the relationship between whole-blood FK506 concentrations and systemic tissue FK506 levels was assessed using Pearson correlation methods, with Fisher’s r to z transformation. All values are expressed as mean ± S.E. p < .05 was regarded as statistically significant.

Results

Skin FK506 Concentrations. Figure 1 shows the skin FK506 levels measured on day 6 in both the right and left forelimbs at each of the three i.a. doses studied. Skin drug concentrations were significantly higher in the locally treated right limb than in the left limb only at the highest dose of 0.20 mg/kg/day, and only a minimal regional advan-
tage of local drug delivery (mean R/L ratios 1.0–1.3) was obtained over the dose range studied (Fig. 2). There was a significant increase in mean FK506 level with dose bilaterally ($p < .001$ in both cases), and left-sided skin FK506 concentrations correlated reasonably well with systemic whole-blood drug levels ($r = 0.75$, $p < .001$). $K_p$ did not vary significantly with dose ($p = 0.09$), with mean values in the 15 to 25 range (Fig. 3).

**Muscle FK506 Concentrations.** Mean muscle FK506 levels significantly increased with dose on both sides (Fig. 4; $p < .001$, bilaterally), but were significantly higher in the right than in the left limb only at 0.1 mg/kg/day. The mean regional advantage achieved again remained relatively constant and negligible over the dose range studied (1.1- to 1.3-fold; Fig. 2). Left-sided muscle FK506 concentrations strongly correlated with systemic blood levels ($r = 0.93$, $p < .001$) and mean $K_p$ did not vary significantly with dose ($p = 0.11$), ranging from 11 to 18 (Fig. 3).

**Bone Marrow FK506 Concentrations.** Marrow FK506 levels were in general 3- to 4-fold lower than those measured in skin and muscle (Fig. 5), and demonstrated significant variation with dose bilaterally (Fig. 6; $p < .001$ in both cases). There were no significant differences between right- and left-sided drug concentrations at any of the three doses, and no meaningful regional advantage was realized (Figs. 2 and 6). Left-sided bone marrow FK506 levels very strongly correlated with blood concentrations ($r = 0.97$, $p < .0001$), and mean $K_p$ values remained in the narrow 4 to 5 range with increasing dose (Fig. 3; $p = 0.50$).

**Bone FK506 Concentrations.** Bone FK506 levels were in general an order of magnitude lower than those measured in skin and muscle (Fig. 5), but similar to those in blood (Fig. 6). While left-sided drug concentrations increased significantly with dose ($p = 0.001$), right-sided levels did not ($p = 0.10$ overall and $p = NS$ for all pairwise dose comparisons) (Fig. 7). Drug concentrations were significantly higher in the right than in the left limb only at the lowest dose of 0.05 mg/kg/day, producing a mean regional advantage of 2.5, versus advantage ratios of 1.4 and 1.3 at 0.10 and 0.20 mg/kg/day, respectively (Figs. 2 and 7). Only a weak, barely significant correlation was observed between left-sided bone FK506 concentrations and systemic whole-blood drug levels ($r = 0.49$, $p = 0.04$) and mean $K_p$ values did not change over the dose range studied (Fig. 3; $p = 0.62$).

**Forelimb Tissue FK506 Concentration Ratios.** Figure 8 illustrates that on the right side, there were no significant variations of skin/muscle, skin/bone, and skin/bone marrow forearm FK506 concentration ratios with increasing dose ($p = 0.24, 0.32$, and 0.22, respectively). Skin/bone and skin/ bone marrow, but not skin/muscle, ratios varied significantly with dose on the left-side ($p = 0.02, 0.04$, and 0.53, respectively). Overall, there were no significant differences between right- and left-sided tissue drug concentration ratios at any of the three doses examined.

**Systemic Tissue FK506 Levels and $K_p$ Values during i.a. Infusion.** Highest concentrations of FK506 were noted in the spleen, lung, and kidney (Fig. 5). In descending order, cardiac drug concentrations were similar to those in skin and skeletal muscle; fat concentrations were similar to those in bone marrow; and liver and blood concentrations were simi-
lar to those in bone. FK506 concentrations increased significantly with dose in all nonlimb tissues \((p < 0.005\) in all cases), except liver \((p = 0.06)\) (Fig. 5). In heart \((p < .0001)\), kidney \((p = 0.05)\), and lung \((p = 0.04)\), mean \(K_p\) values significantly decreased with increasing dose, whereas in liver \((p = 0.22)\), spleen \((p = 0.14)\), and fat \((p = 0.72)\), \(K_p\) values remained relatively stable over the dose range studied (Fig. 9).

**Discussion**

Despite continuous administration of FK506 directly into the artery supplying the rabbit forelimb at doses producing mean systemic blood levels just below and well into the therapeutic range \((10–20\, \text{ng/ml})\), we were unable to demonstrate a clinically significant regional pharmacokinetic advantage of local drug delivery in any of the limb tissue components studied. This observation is in marked contrast to our previous results with i.a. CSA infusion in the same model, in which impressive right/left concentration gradients were produced in skin \((4- to 14\)-fold), muscle \((3- to 9\)-fold), bone \((3- to 4\)-fold), and bone marrow \((3- to 9\)-fold) at all except the lowest of five doses studied (Shirbacheh et al., 1999a,b).

Several other differences between our results with the two immunosuppressants are also noteworthy. First, the relative distribution of drug among the locally and systemically treated limb tissues was very different. In the case of CSA,
bone marrow drug concentrations were 5- to 10-fold higher than those in skin, and skin concentrations were generally 2- to 3-fold higher than those in muscle and bone (Shirbacheh et al., 1999a,b). In contrast, FK506 levels in skin were equivalent to those in muscle, 3- to 4-fold those in bone marrow, and 10- to 20-fold those in bone. Table 1 compares the range of mean $K_p$ values noted for each systemically treated limb tissue during multidose FK506 infusion in the present study with those noted during multidose CSA infusion in our previous work (Shirbacheh et al., 1999a). With both drugs, $K_p$ did not vary significantly with dose in any of the four tissues studied, except for CSA-treated bone. In this case, $K_p$ values significantly decreased over the dose range studied, suggesting that saturation of bone uptake may be occurring, even at relatively low systemic whole-blood CSA concentrations (Shirbacheh et al., 1999a). Second, locally treated skin and bone appeared to rapidly saturate with CSA, reaching near-maximal concentrations at relatively low i.a. doses (Shirbacheh et al., 1999a), whereas FK506 levels increased steadily with increasing i.a. dose in all locally treated tissues. Finally, although the highest CSA concentrations were also found in kidney, lung, and heart among the various nonlimb tissues examined (Shirbacheh et al., 1999b), liver CSA concentrations were higher than those of skin and muscle, whereas liver FK506 concentrations were lower than those of skin and muscle.

One factor that may be responsible in large part for the observed pharmacokinetic differences between FK506 and CSA in our model is the markedly different distribution of each drug within whole blood. FK506 is rapidly absorbed and strongly retained by red blood cells (RBCs) (Sewing, 1994; Kelly et al., 1995), which have been demonstrated to contain a cytoplasmic, high-affinity FK506 binding protein (Kay et al., 1991). As a result, whole blood/plasma ratios range between 10 and 40 in healthy subjects (Habucky et al., 1991) as well as in transplant patients (Japanese FK506 Study Group, 1991; Venkataramanan et al., 1991). For example, Nagase et al. (1994) found that at clinically relevant whole-blood drug levels of 5 to 50 ng/ml, FK506 is 94 to 98% within erythrocytes in human blood, with only 1 to 2% in plasma. These values are in marked contrast to those previously reported for CSA, in which case 58% of circulating drug is bound to RBCs and 33% is in plasma (LeMaire and Tillement, 1982), with whole blood/plasma ratios approximately 2.0 in healthy subjects and 1.3 to 1.4 in renal and hepatic allograft recipients (Ptachcinski et al., 1986). Interestingly, the distribution of FK506 in whole blood in rabbits, unlike that in rats (Nagase et al., 1994; Iwasaki et al., 1991), has been found to be similar to that in human, with 74.3% of drug bound to RBCs, 18.1% in plasma, and 6.9% bound to mononucleocytes (Piekoszewski et al., 1993). Indeed, Piekoszewski et al. (1993) examined the disposition of FK506 in male New Zealand rabbits with specific regard to the role of RBC binding in hepatic clearance, and noted that nonlinear, strong RBC binding occurs, with high whole blood/plasma ratios (~9) at low (therapeutic) plasma FK506 concentrations, and slow diffusion of drug from erythrocytes to plasma ($T_{1/2} = 7$ min). These authors also concluded that because FK506 disposition and distribution was similar to that observed in humans, the rabbit can be used as a reasonable model for pharmacokinetic studies of the drug. Based on the above information, we hypothesize that failure to achieve a sizable local-to-systemic tissue drug concentration gradient during extremity FK506 infusion in our model is due to the fact that as FK506 is continuously delivered to the arterial blood supply of the limb, it is strongly bound by erythrocytes and is released too slowly to the plasma to get into the tissue as free drug given the relatively long RBC/plasma equilibration time relative to tissue transit time (Venkataramanan et al., 1995). Along these lines, a regional pharmacokinetic advantage is attainable only during the first passage of drug through the target organ (Eckman et al., 1974; Collins, 1984). Once the drug enters the venous effluent of the limb, it is distributed as although injected i.v., with plenty of time for RBC/plasma equilibration to occur as steady state is reached.

According to our hypothesis, one should be able to increase the i.a. FK506 dose to such a degree so as to saturate RBC binding and drive drug into the plasma and hence into the tissue to ultimately achieve a regional advantage in our model. In the case of CSA, this “break point” occurred when the local dose was increased from 1.0 to 2.0 mg/kg/day, making it possible to achieve a significant regional pharmacokinetic benefit at a dose producing subtherapeutic systemic drug levels (Shirbacheh et al., 1999a). However, because FK506 is much more extensively distributed to RBCs than is CSA, it is not surprising that we were unable to realize a regional advantage even at a dose (0.2 mg/kg/day) already producing what would be considered supratherapeutic whole-blood drug concentrations (mean 24 ± 5 ng/ml). Clearly, achieving a regional advantage at the expense of systemic toxicity would not be a desirable overall outcome.

The “negative” results with FK506 presented herein, when taken together with our previous “positive” results with CSA, contribute important information with regard to establishing a set of desirable characteristics that an immunosuppressant should possess to be used in our vascularly isolated rabbit forelimb model to pharmacokinetic, and perhaps ultimately, therapeutic, benefit. Clearly, the simple expression often used to define and calculate regional advantage, $1 + \frac{C_l}{Q_T}$, where $C_l$ is the systemic clearance of drug outside the target organ calculated during i.v. administration, and $Q_T$ is blood flow to the target organ (Collins, 1984), is not in and of itself a reliable predictor, in that it does not account for other parameters such as variation in whole-blood drug distribution and saturation of RBC, plasma, or tissue protein binding and carrier transport systems. For example, when comparing mean values for total body clearance in whole blood obtained from three-compartment pharmacokinetic analysis following i.v. bolus administration of both CSA 5 mg/kg (407 ml/h/kg; Awni and Sawchuk, 1985) and FK506 0.5 mg/kg (312 ml/h/kg; Piekoszewski et al., 1993) to male New Zealand white rabbits, one would have expected to find only a marginal increase in pharmacokinetic advantage achievable with ex-
tremity i.a. CSA infusion when compared with FK506 infusion. The fact that a large difference was noted between these two agents in the rabbit model suggests that the degree of RBC binding and rate of drug efflux from erythrocytes may be important considerations with regard to total drug delivery and pharmacokinetic advantage when selecting an immunosuppressive agent for local perfusion of limb allografts.

To our knowledge, the generalized tissue distribution of FK506 within rabbit tissues following IV bolus or during continuous i.a. or i.v. administration has not been previously reported. Our findings are similar to those observed in the rat by Venkataramanan et al. (1990) following i.m. administration of FK506 (1.28 mg/kg/day) for 2 weeks and in the Cynomolgus monkey by Wijnen et al. (1991) following oral administration (1 and 10 mg/kg/day) for 90 days. In these studies, highest drug concentrations were found in lung, kidney, spleen, heart, and muscle, with liver levels being equivalent to those in plasma.

In conclusion, pharmacokinetic studies of i.a. FK506 infusion performed at multiple dose levels in our novel rabbit model failed to demonstrate a clinically significant regional advantage of local drug delivery in any of the four limb tissue components studied. This observation is in marked contrast to our previous results with i.a. CSA infusion in the same model, in which 3- to 14-fold right/left concentration gradients were produced in skin, muscle, bone, and bone marrow. We hypothesize that, in addition to the well-established parameters of total body clearance and target organ blood flow, the degree of RBC binding and rate of drug efflux from erythrocytes may be important considerations with regard to optimizing tissue drug delivery and pharmacokinetic advantage when selecting an immunosuppressive agent for local perfusion of limb allografts. Our findings suggest that, despite its demonstrated efficacy in experimental and clinical transplantation, FK506 would not be an appropriate immunosuppressant to deliver via the i.a. route for prevention of limb allograft rejection.

References


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