Pharmacokinetic Advantage of Intra-arterial Cyclosporin A Delivery to Vascularly Isolated Rabbit Forelimb. II. Dose Dependence

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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. cyclosporin A (CSA) administration. CSA was infused continuously via osmotic minipump into the right brachial artery of New Zealand rabbits at multiple doses ranging from 1.0 to 8.0 mg/kg/day. On day 6, CSA concentrations were measured in aortic whole blood, as well as in skin, muscle, bone, and bone marrow samples from both right and left forelimbs. The variation of right-sided mean CSA concentrations with dose was tissue dependent and saturable in the case of skin and bone, whereas left-sided tissue concentrations correlated significantly with systemic blood levels. At 1.0 mg/kg/day, there were no significant differences between right and left mean CSA concentrations for all four tissues examined. However, with a doubling of the i.a. dose, huge increases in local tissue CSA concentrations were produced with only very modest increases in systemic whole-blood and tissue drug levels, resulting in a 4-fold regional advantage (right/left ratio of CSA concentrations) in bone and bone marrow, 7-fold in muscle, and 14-fold in skin. With further dose increases to 8.0 mg/kg/day, the regional advantage decreased to 4-fold in skin, increased to 9-fold in bone marrow, remained relatively constant in bone, and initially decreased and then increased to 9-fold in muscle. These favorable pharmacokinetic results suggest that reduced, local doses of CSA might be useful in preventing extremity composite tissue allograft rejection with decreased systemic drug exposure.

Effective antirejection therapy with minimal systemic morbidity is required if limb transplantation is to become a clinical reality. 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 for rejection prophylaxis with decreased systemic drug exposure. Along these lines, we developed a novel rabbit forelimb 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., 1999). In initial studies, we demonstrated that a sizable, 4- to 7-fold regional pharmacokinetic advantage could be achieved in locally treated skin, muscle, bone, and bone marrow during single-dose, i.a. cyclosporin A (CSA) infusion when compared with tissues in the contralateral limb or in the limbs of animals receiving same-dose i.v. therapy.

One factor known to diminish the expected gain from i.a. infusion is nonlinearity in pharmacokinetics as a result of saturation of carrier systems for transfer of free drug into the target organ (Smits and Thijssen, 1987). It is certainly conceivable that at the single dose chosen for previous study (4.0 mg/kg/day), some or all of the tissue components of the locally treated limb were already saturated with CSA, so that lowering the i.a. dose would not significantly reduce local tissue levels, but might have significantly reduced systemic whole-blood and tissue levels, thereby increasing regional advantage. In an effort to further address this issue, the primary

**ABBREVIATIONS:** $C_{\text{systemic(i.a.)}}$, systemic drug concentration at steady state during i.a. infusion; $C_{\text{target(i.a.)}}$, steady-state drug concentration in target organ during i.a. infusion; $C_{\text{target(i.v.)}}$, steady-state drug concentration in target organ during i.v. infusion; $C_{\text{l}}$, systemic clearance; CSA, cyclosporin A; CTA, composite tissue allograft; inf, constant drug infusion rate; $K_{\mu}$, equilibrium distribution ratio; $R_{\text{target}}$, regional advantage; $Q_{\text{T}}$, target organ blood flow.
goals of the present study were to 1) examine the variation of CSA tissue concentrations with dose in both locally treated and systemically treated (contralateral) limbs, 2) determine the dose dependence of the regional advantage attainable in various limb tissues during i.a. CSA infusion in relation to systemic blood levels, and 3) examine the dose dependence of the equilibrium distribution ratio for systemically treated limb tissues during i.a. CSA infusion.

Materials and Methods

Animals. Thirty-eight 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 Louisville 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 forelimb model of local immunosuppression as described in detail previously (Shirbacheh et al., 1999). In brief, the distal end of a composite Intramedic PE-60/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 mid-arm level was performed to eliminate collateral circulation and simulate allografting. Pumps were filled with CSA solution (Sandimmune, 50 mg/ml; Sandoz Pharmaceuticals Corp., East Hanover, NJ) and diluted with vehicle (Cremophor EL), if necessary, to achieve final concentrations of 50, 25, or 12.5 mg/ml.

Pharmacokinetic Study. CSA was administered by continuous i.a. infusion at 1.0 (n = 6), 2.0 (n = 8), 3.0 (n = 5), 4.0 (n = 12), and 8.0 (n = 7) mg/kg/day. To administer the highest dose, two pumps were implanted and their catheters joined via a Y-connector. In six of the eight animals receiving 2.0 mg/kg/day, skin biopsies from right and left forelimbs as well as jugular venous blood were obtained on days 1, 3, and 5 for determination of CSA levels. On postoperative day 6, animals were anesthetized with an i.m. injection of ketamine (37.5 mg/kg) and xylazine (5 mg/kg). Following local thoracotomy, a blood sample was drawn from the aorta for determination of whole-blood CSA concentration, the aorta was transected, and the animal euthanized by bleeding. Skin, muscle, bone, and bone marrow tissue samples were obtained symmetrically from both right and left forelimbs for determination of CSA levels. All samples were stored at −80°C until analysis.

CSA Assay. Whole-blood CSA concentrations were determined using the enzyme-multiplied immunoassay specific method (Beresini et al., 1993) on the Cobas Mira chemistry analyzer. Tissue specimens were extracted into methanol before analysis for CSA using the enzyme-multiplied immunoassay as described previously (Shirbacheh et al., 1999).

Data Analysis. Right (locally treated) and left (systemically treated) forearm tissue CSA levels were compared at each dose level using a paired Student's t test. The regional pharmacokinetic advantage of i.a. CSA infusion for each tissue at each dose was determined from the right/left (R/L) ratio of tissue CSA concentrations. The equilibrium distribution ratio (Kₑ) was calculated for each tissue at each dose from the left-sided tissue-to-whole-blood CSA concentration ratio (Bernareggi and Rowland, 1991). A one-way ANOVA was used to determine whether right- and left-sided tissue CSA concentrations and left-sided Kₑ values were related to local CSA dose. The one-way ANOVA was also used to determine whether bilateral skin and whole-blood CSA concentrations varied with time over the 6-day infusion period in the subgroup of six rabbits receiving 2.0 mg/kg/day i.a. In both cases, the Scheffe's F procedure was used for post hoc, multiple, paired comparisons. The linearity of the relationship between whole-blood CSA concentrations and systemic tissue CSA 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 CSA Concentrations. Figure 1 gives the skin CSA levels measured on day 6 in both the right and left forelimbs at each of the five i.a. doses studied. Skin drug concentrations were significantly higher in the locally treated right limb than in the left limb, except at the lowest dose of 1.0 mg/kg/day. Although there was a significant variation of mean CSA level with dose bilaterally (p < .0001 in both cases), the nature of the variation differed somewhat between the two sides. On the left side, skin CSA concentrations gradually increased with increasing dose and strongly correlated with systemic whole-blood drug levels (Fig. 2; r = 0.82, p < .0001). Variation of Kₑ with dose was statistically significant solely due to the peak observed at 3.0 mg/kg/day (Fig. 3; p = 0.001), but otherwise remained stable, with mean values in the 2.5 to 3.5 range. In contrast, on the right side, mean skin CSA concentrations significantly increased 13-fold when the i.a. dose was increased from 1.0 to 2.0 mg/kg/day (1.0 versus 2.0; p = 0.004), remained at this high level when the dose was further increased to 3.0 and 8.0 mg/kg/day (2.0 versus 3.0, 2.0 versus 8.0, and 3.0 versus 8.0; p = N.S.), but fell to an intermediate level at 4.0 mg/kg/day (3.0 versus 4.0, 4.0 versus 8.0; p < .01) (Fig. 1). As a consequence, the mean R/L ratio, or regional advantage of local drug delivery, was minimal (1.3-fold) at 1.0 mg/kg/day, maximal (14-fold) at 2.0 mg/kg/day, and decreased to 4-fold at the upper end of the dose range studied (Fig. 4).

Muscle CSA Concentrations. Mean muscle CSA levels were significantly higher in the right than in the left limb at 2.0, 4.0, and 8.0 mg/kg/day, and varied significantly with dose on both sides (Fig. 5; p < .0001, bilaterally). Again, on the left side, muscle CSA concentrations gradually increased with increasing dose and significantly correlated with systemic whole-blood drug levels (Fig. 2; r = 0.60, p < .0001). Overall, mean Kₑ did not vary significantly with dose and remained in the narrow 1.2 to 1.7 range, except for the increase at 3.0 mg/kg/day (Fig. 3; p = N.S. for all pairwise dose comparisons). On the right side, mean muscle CSA concentrations increased 5-fold when the i.a. dose was in-

Fig. 1. Day 6 skin CSA concentrations in the right (R) and left (L) forelimbs during continuous i.a. infusion at doses ranging from 1.0 to 8.0 mg/kg/day. Values plotted are mean ± S.E.
increased from 1.0 to 2.0 mg/kg/day \( (p = \text{N.S.}) \), remained at this intermediate level when the dose was further increased to 3.0 and 4.0 mg/kg/day \( (p = \text{N.S. for all 1.0–4.0 pairwise dose comparisons}) \), and significantly increased another 4-fold with a dose increase to 8.0 mg/kg/day \( (p < .005 \text{ for all pairwise dose comparisons with 8.0}) \) (Fig. 5). As a result, mean regional advantage ranged from 2- to 9-fold, with maximal values at 2.0 and 8.0 mg/kg/day (Fig. 4).

**Bone Marrow CSA Concentrations.** Marrow CSA levels were in general an order of magnitude higher than those measured in the other limb tissues, and were significantly greater in the locally treated limb at 4.0 and 8.0 mg/kg/day (Fig. 6). Mean drug concentrations steadily increased with increasing dose bilaterally \( (p < .0005) \), and left-sided levels correlated significantly with systemic whole-blood drug levels (Fig. 2; \( r = 0.60, p < .0001 \)). Mean \( K_p \) values remained stable in the 14 to 20 range with increasing dose (Fig. 3; \( p = \text{N.S.} \)). Mean regional advantage steadily increased from 1- to 9-fold over the dose range studied (Fig. 4).

**Bone CSA Concentrations.** Bone CSA levels were significantly higher in the right than in the left limb at 2.0, 4.0, and 8.0 mg/kg/day (Fig. 7). Although the overall variation of mean drug concentration with dose achieved statistical significance on both right and left sides \( (p = 0.01 \text{ and } 0.003, \text{respectively}) \), the nature of this relationship was much less apparent when compared with that observed in the other limb tissues. On both sides, no pairwise dose comparisons of mean drug concentration were significant, and only a weak correlation was observed between bone CSA concentrations and systemic whole-blood drug levels (Fig. 2; \( r = 0.43, p = 0.009 \)). Interestingly, the variation in \( K_p \) with dose was highly significant (Fig. 3; \( p = 0.0002 \)), with a gradual decrease in mean values from 4.5 at 2.0 mg/kg/day to 1.2 at 8.0 mg/kg/day. Aside from the lowest dose, mean regional advantage remained fairly constant in the 3- to 4-fold range (Fig. 4).
The regional pharmacokinetic advantage of i.a. drug administration ($R_{\text{target}}$) is defined by the ratio $C_{\text{target(i.a.)}}/C_{\text{target(i.v.)}}$, where $C_{\text{target(i.a.)}}$ is the steady-state drug concentration in the target organ during i.a. infusion and $C_{\text{target(i.v.)}}$ is the steady-state drug concentration in the target organ during i.v. administration (Collins, 1984). In our initial studies performed using a CSA dose of 4.0 mg/kg/day (Shirbacheh et al., 1999), we found that tissue drug concentrations in the systemically treated left limb of animals receiving i.a. infusion were equivalent to those achieved in the limbs of animals receiving i.v. infusion, confirming that there was no first-pass extraction of CSA by the locally treated limb. Therefore, in the current study, it was not necessary to perform more labor-intensive, double-pump, sequential i.a. and i.v. infusion studies in the same set of animals to calculate $R_{\text{target}}$ at each dose level, as described in previous work examining the pharmacokinetics of i.a. drug delivery in a canine renal autotransplant model (Gruber et al., 1990, 1992). Rather, we felt it simpler to use the $R$ (local)/$L$ (systemic) tissue concentration ratios obtained during i.a. infusion and eliminate the need for same-dose i.v. groups entirely. Bilateral mean tissue CSA concentrations and consequently, R/L tissue concentration ratios, in the 12 animals receiving 4.0 mg/kg/day in the current study were essentially equivalent to values previously reported for seven rabbits in our initial study (Shirbacheh et al., 1999), attesting to the reproducibility of results in our model.

As noted, the ratio given above for $R_{\text{target}}$ is only valid after steady state has been reached (Smits and Thijsse, 1987). The results of our pilot study examining the time course of CSA accumulation in rabbits receiving 2.0 mg/kg/day indicate that steady-state levels are achieved in whole-blood and systemically treated skin within 72 h and in locally treated skin by 120 h. These pharmacokinetic results are in agreement with those previously obtained by Awni and Sawchuk (1985) in the male New Zealand White rabbit and Bernareggi and Rowland (1991) in rodents following continuous i.v. and s.c. CSA infusion, respectively (Shirbacheh et al., 1999).

Therefore, we are reasonably certain that steady state was reached in our model in whole blood as well as in each limb tissue component by the day 6 endpoint, and that our determinations of $R_{\text{target}}$ are valid.

Assuming steady-state conditions and linear pharmacokinetics without saturation of drug transport processes into or elimination processes from the target organ, $R_{\text{target}}$ is not dose dependent, and may also be calculated from the expression $1 + C_{l}/Q_{\text{t}}$, where $C_{l}$ is the systemic clearance of drug outside the target organ calculated during i.v. administration, and $Q_{\text{t}}$ is blood flow to the target organ (Eckman et al., 1974; Collins, 1984; Smits and Thijsse, 1987). After i.v. administration of 5 mg/kg CSA to New Zealand rabbits, Awni and Sawchuk (1985) determined mean total body clearance to be 6.8 ml/min/kg using a three-compartment open model and 13.3 ml/min/kg using model-independent methodology. We have previously determined brachial artery blood flow to be 2.3 ± 0.1 ml/min by electromagnetic probe measurement in 18 healthy male New Zealand rabbits of the same weight as those used in our study. Substituting these values into the expression $(1 + C_{l}/Q_{\text{t}})$ given above yields a predicted $R_{\text{target}}$ in the 9 to 18 range for i.a. versus i.v. CSA delivery. As long as there is no influence of i.a. CSA infusion on limb blood flow and no metabolism of CSA within limb tissue components, this predicted range for $R_{\text{target}}$ represents the maximal regional pharmacokinetic advantage achievable in the absence of saturation kinetics.

Indeed, it is interesting to note that in our study, the maximal mean $R_{\text{target}}$ values obtained for skin (14), muscle (9), and bone marrow (9) were all within or approached the predicted range, but that for bone (4) did not. Clearly, the limb tissue components differ with regard to both their affinity and capacity for binding locally administered CSA. Skin and bone appear to rapidly saturate with CSA at relatively low (2.0–3.0 mg/kg/day) i.a. doses, whereas bone marrow has an enormous capacity to bind CSA, presumably because of its high fat content (Bäckman et al., 1988), resulting in drug concentrations that are an order of magnitude higher than those in the other tissues and that steadily increase with dose. Along these lines, the overall variation of right-sided limb tissue drug concentrations over the dose range studied suggests that, with increasing dose, a
greater proportion of locally delivered CSA is “driven” into the bone marrow as the other tissues become saturated due to more limited capacity for binding drug.

In skin, muscle, and bone marrow, left-sided CSA concentrations strongly correlated with systemic whole-blood drug levels, with mean \( K_p \) values remaining stable over the dose range studied. In contrast, left-sided bone CSA concentrations only weakly correlated with blood levels, and \( K_p \) values significantly decreased over the 2.0 to 8.0 mg/kg/day dose range, suggesting that saturation of tissue uptake may be occurring, even at relatively low systemic whole-blood drug concentrations. It is noteworthy that the range of mean \( K_p \) values we determined for skin (2.5–3.5), muscle (1.2–1.7), and bone (1.2–4.5) are all similar to those reported by Bernareggi and Rowland (1991) for corresponding tissues in rats (3.0–3.9, 1.3–2.4, and 2.5–3.4, respectively) following 6 days of continuous, pump-based s.c. CSA infusion at 2.7 and 13.9 mg/kg/day.

At the lowest dose of 1.0 mg/kg/day, there were no significant differences between right and left mean CSA concentrations for all four tissues examined, and consequently, only a minimal regional advantage was realized. However, with a doubling of the i.a. infusion rate to 2.0 mg/kg/day, huge (5- to 13-fold) increases in local tissue mean CSA concentrations were produced with only very modest increases in systemic whole-blood and tissue drug levels, resulting in a 4-fold regional advantage in bone and bone marrow, 7-fold in muscle, and 14-fold in skin. The explanation for our failure to achieve a substantial advantage at 1.0 mg/kg/day and the “threshold effect” observed in \( \text{R}_{\text{target}} \) between 1.0 and 2.0 mg/kg/day is not entirely clear. The steady-state CSA concentration in the brachial artery during i.a. infusion is given by the sum of two components: \( \text{inf}/Q_T + \text{CSA}_{\text{systemic}} \) (i.a.), where \( \text{inf} \) is the constant infusion rate of drug and \( \text{CSA}_{\text{systemic}} \) (i.a.) is the concentration of drug present in the blood returning to the limb at steady state (Collins, 1984). Using our measured mean value of 84 ng/ml for \( \text{CSA}_{\text{systemic}} \) (i.a.), with \( \text{inf} = 3 \) mg/day and \( Q_T = 2.3 \) ml/min, yields an estimated local CSA concentration of 900 ng/ml in whole blood at steady-state during i.a. infusion at 1.0 mg/kg/day. This calculated value is 10-fold higher than that present in the contralateral limb at the same dose (84 ng/ml), but is not producing higher tissue concentrations. Moreover, the estimated local whole-blood CSA concentration at 1.0 mg/kg/day is even greater than that present in the contralateral limb at an 8-fold higher dose (mean 547 ng/ml), although lower tissue drug levels are achieved (compare right-sided tissue levels at 1.0 mg/kg/day with left-sided levels at 8.0 mg/kg/day in Figs. 1, 5, 6, and 7). We hypothesize that, during the very brief period of time in which the blood components are exposed to high concentrations of CSA in the locally treated right limb at 1.0 mg/kg/day, the rate of drug equilibration between red blood cells and plasma must not be rapid enough to promote transport/passage diffusion of the free fraction from the vascular/interstitial compartment to the intracellular space and thereby increase tissue levels (Smits and Thijssen, 1987; Dedrick, 1988).

In both small- and large-animal models, it has become clear that vascularized composite tissue allografts (CTAs) elicit nonsynchronized immune responses of varying intensity among their tissue components, with skin and muscle being the most antigenic, bone of intermediate immunogenicity, and cartilage and tendon the least antigenic (Daniel et al., 1986; Press et al., 1986; Black et al., 1988; Doi et al., 1989; Hotokebuchi et al., 1989; Stevens et al., 1990; Tan et al., 1991; and Benhaim et al., 1993). It is therefore very intriguing that low-dose (2.0 mg/kg/day) extremity CSA infusion produced a huge (14-fold) regional advantage in skin, a large (7-fold) advantage in muscle, and significant (4-fold) advantages in bone and bone marrow in the presence of low systemic blood levels, thereby “matching” relative immunogenicity with relative tissue drug concentration.

In conclusion, pharmacokinetic studies of i.a. CSA infusion performed at multiple dose levels in our novel rabbit model suggest that reduced local doses of CSA might be useful in preventing extremity CTA rejection with decreased systemic drug exposure and toxicity. Whether the pharmacokinetic advantage of regional CSA delivery can be converted to a therapeutic advantage in future antirejection efficacy and toxicity studies of local immunosuppression in large-animal extremity CTA models remains to be elucidated.

References


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