Influence of Hepatic Ischemia-Reperfusion Injury on Tacrolimus Acute Renal Toxicity in Pigs

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THE DEVELOPMENT of new immunosuppressants has been a determining factor in the recent surge in organ transplants. Tacrolimus, which was introduced in clinical practice by Starzl in 1989, has proved to be useful for prevention and treatment of rejection.[1–5] Among its side effects, nephrotoxicity is of particular importance. The precipitating causes of acute nephrotoxicity (ANT), which may affect 35% to 45% of liver transplant patients are uncertain,[5–8] but possibly include early graft malfunction, a phenomenon related to ischemia and reperfusion injuries, which result at least in part from shortcomings in the organ preservation.[5–9] To shed light on the relationship between ischemic liver injury and ANT caused by tacrolimus, we designed a comparative experimental study of the kidney damage generated in two experimental models of hepatic ischemia-reperfusion injury: non–heart-beating donor and an ischemia–reperfusion model with the organ in situ.

MATERIAL AND METHODS

Experimental Design

Thirteen large white Landrace pigs weighing around 30 kg were divided among three experimental groups.

(1) Liver Transplant From Donor With Cardiac Arrest (LT group, $n = 5$)

Once the hepatic pedicle (HEP) structures had been dissected, we administered 90 mg of calcium heparin IV, and 10 minutes later 40 mg of potassium chloride which caused the animal's death. After 60 minutes, we clamped the thoracic aorta and proceeded to perform perfusion with 1 I of University of Wisconsin solution (4°C) via the infrarenal aorta in retrograde fashion. During back-table surgery, we perfused 1 I of University of Wisconsin solution through the porta. The organ was stored at 2 to 4°C for 60 minutes. The implants were performed according to the procedure described by Starzl using shunting of the portal flow by a passive portojugular shunt. The biliary drainage reconstructed using an end-to-end choledocho-choledochostomy.

(2) In Situ Ischemia Group Nontreated With Tacrolimus (IS Group, $n = 4$)

We dissected the HEP structures and the left external jugular vein, and freed the splenic vein close to the hilar area, after insertion of heparinized Gott no. 22 cannulae in the splenic and left external jugular veins to construct a passive splenojugular shunt. We then proceeded immediately to clamp the elements of the hepatic pedicle and infrahepatic vena cava (IHVC) at a point proximal to the renal veins. The flow in the IHVC was maintained without interruption for 60 minutes (mean duration of the anhepatic phase in the liver transplantation model), whereas the clamp was kept on the HEP for 160 minutes.

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(3) IS-Tacrolimus Group (n = 4): In Situ Ischemia Model Treated With Tacrolimus

Administration Protocol for Tacrolimus. The animals belonging to the HF and IS-Tacrolimus group received a daily dose of 0.05 mg/kg of Tacrolimus (Prograf, Fujisawa Pharmaceutical Co Ltd, Osaka, Japan) IM for 3 days prior to the experiment and when anesthesia was induced. In LT group, the treatment was given to both donor and recipient. Tacrolimus blood concentrations were measured at the time of induction of anesthesia and 24 hours after revascularization.

Blood Samples

Venous and arterial blood samples were obtained at the time of induction of anesthesia, at the end of the hepatic ischemia period (EWI) in the LT group; and at the end of the anhepatic phase (EAP) in the HF model. In addition to this, samples were taken at 1, 4, and 24 hours after revascularization. The damage caused by an ischemia–reperfusion injury and the overall liver function were determined by quantification of GOT, GPT, LDH, total billirubin, direct bilirubin, total proteins, albumin, and prothrombin time. Yidney function was assessed by monitoring diuresis and serum creatinine levels.

Biopsies

Liver biopsies were taken at the following points: baseline, prerevascularization, 1 hour, and 24 hours after revascularization or at the moment of death. In the IS model we performed a right kidney biopsy by tru-cut at laparotomy, at the end of the clamping of the vena cava, at the end of clamping of the hepatic pedicle and after 1 hour. The survivors were killed on the 7th day after surgery. At the moment of death or slaughter we took a biopsy from the left kidney.

Statistical Study

The statistical analysis was performed using SPSS 9.0. We used Kruskall-Wallis test to assess differences in albumin levels, GOT, GPT, creatinine, and prothrombin time between groups. We applied paired comparisons for homogeneous variances: post-hoc tests using the methods of Scheffe~ and Bonferroni. We compared the baseline and final creatinine levels in each of the groups and assessed significance using the ranked test with Wilcoxon’s sign, a nonparametric test for related samples which compares the medians of the baseline and final creatinine levels.

RESULTS

Liver Function

We observed no significant differences in any of the biochemical parameters related to liver function, and therefore assume that similar ischemic damage was generated in the three groups (Table 1). The GOT began to rise from the end of the ischemic period onward; the maximum concentration was noted at 24 hours after revascularization: the mean values for each group were 1296 ± 867, 1157 ± 465, and 1802 ± 1783 U/I (P = .676), respectively. The maximum GPT levels were also achieved at 24 hours after revascularization, with mean values for each group of 81 ± 27, 122 ± 43, and 136 ± 133 U/I (P = .567), respectively. The rise in the LDH levels began later, although first noted in the 1 hour samples, they also peaked at 24 hours with mean values of 2970 ± 985, 2514 ± 1049, and 3299 ± 3643 U/I (P = .529), respectively. The breadth of the range of values in group III was due to the congestive damage that the organ experienced during the phase in which the pedicle was clamped, although the cause of this effect could not be precisely determined, we suspect right ventricular failure as a result of hydric overload. The drop in

prothrombin activity, which began at the end of the ischemic phase, reached its model at 4 hours. From then on, gradual recovery was noted, with normal figures being reached by 24 hours. The mean values at 4 hours were 59.1 ± 16%, 76.5 ± 42%, and 78.8 ± 15% (P = .77), respectively. The albumin concentration began to fall at the end of the ischemic period, with the lowest values at 4 hours when a slight recovery set in. No differences between groups were found in any of the tests: the mean values at 4 hours were 1.2 ± 0.33, 1.7 ± 0.6, and 1.7 ± .3 g/dL (P = .197) respectively. The values for GGT, FA, BT, BD, PT, TCK, and FNG did not show significant differences at any time during the study.

Tacrolimus Levels

The baseline levels in animals of groups I and III after 3 days of treatment and before the experiment were always within the therapeutic range: 6.45 ± 1.3 and 8.1 ± 1.75 ng/mL (P = .1). Drug concentrations at 24 hours were close to the upper limit of the therapeutic range, but there were no significant differences: 15.2 ± 2.4 and 12.3 ± 5.4 ng/mL (P = .4).

Kidney Function

Baseline kidney function was within normal limits in all three groups. We noted an increase in creatinine values of similar intensity in all three groups from 4 hours onward: 2.18 ± 1, 2.6 ± 0.6, and 2.3 ± 0.7 mg/dL (P = .58). Animals in groups II and III maintained similar levels until 24 hours, whereas those in group III developed rapid renal dysfunction, which led to death from hyperkalemia (mean K^+ = 11.2 ± 2.5 mEq/L). Significant differences were found in the creatinine values at 24 hours: 4.8 ± 1.4, 2.5 ± 1.0 and 2.3 ± 0.8 mg/dL (P = .035 versus group II) (P = .009 versus group III) (Fig 1).

Ischemic Periods

The mean duration of the warm ischemia period (the sum of the time of cardiac arrest plus rewarming) in group I was 176 ± 13 minutes. The grafts were stored cold for 60 minutes, the time mandated in the experimental design. The mean duration of portal ischemia was 184 ± 16 minutes in group I and 170 minutes in groups II and III. The anhepatic phase was prolonged for a period of 59.6 ± 11.6 minutes, a figure taken as a reference to establish the clamping time of IHVC in the model of in situ ischemia.

Pathology Study

The most frequent and striking microscopic finding was the presence in the tubular lumina of dense, amorphous eosinophilic matter arranged either in the form of small rounded drops (mainly in the control specimens) or as diffuse, retracted accumulations giving a vacuole-like appearance. The glomeruli and arteries were well-conserved. The only feature of note in the renal interstices was the presence of small lymphoplasmocytic aggregates, which were characteristically bilateral and situated in the corticomedullary transition zone. No images of tubular fibrosis or necrosis were observed. In the baseline biopsies of the tubules of all groups, particularly the proximal ones, we observed clearing of the epithelial cells because of the presence of large vacuoles of different sizes in the cytoplasm. These vacuoles were either distributed diffusely or else confined to the subcapsular tubules. The presence of small confluent vacuoles of homogeneous sizes characterizes isometric vacuolization. These vacuoles tend to give rise to other, larger ones, which are also homogeneous and occupied the whole cytoplasm. Such vacuoles could be found in a high intensity in almost 100% of the proximal tubules of one of the control group, and were found in moderate intensity in the rest of this group. In groups IS and IS-Tacrolimus, this isometric vacuolization only appeared to be mild. Some specimens showed a marked loss of structure, with images of

fading in the epithelial cells. In the study of the liver specimens, as in the kidney tubules, we observed the presence of amorphous, eosinophilic, proteinaceous material in the sinusoids, which was less dense than that in the tubules. None of the cases showed signs of acute liver rejection.

DISCUSSION

Tacrolimus, an immunosuppressive macrolid that belongs to the family of the calcineurin inhibitors, has been shown to display greater immunosuppressive potency than cyclosporine a, with a lower incidence of episodes of rejection[1–5] and a high degree of efficacy for the treatment of non-ductopenic steroid-resistant rejection.[10] However, tacrolimus does have toxic features common to this group of drugs: neurotoxicity, hyperglycemia, hypertrophy, development of lymphoid and skin tumors, and nephrotoxicity. Renal toxicity caused by tacrolimus is expressed in a well-known series of functional and structural disorders. Decreases in the glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) have been described both in experimental studies[11–13] and in transplant patients.[14] However, a comparative study between the kidney function of patients with terminal hepatopathy (awaiting transplant) and patients who have already received a liver transplant and are being treated with tacrolimus suggest that this drug only increases the ERPF deficit by 7%, and does not reduce the GFR. It would therefore seem that the main factor in kidney deterioration is the hepatopathy itself.[15] These results suggest that the mechanism of kidney dysfunction is mainly vascular, although a toxic effect on the epithelial cells cannot be ruled out.[17] A direct effect of arteriolar vasoconstriction has been demonstrated,[17] which involves a reduction in PGI2 synthesis and an increase in endothelin production.[18,19] Tacrolimus has been shown to perturb the homeostasis of intracellular calcium in smooth muscle via the drug–calcineurin complex, encouraging arteriolar constriction; in fact, the administration of calcium antagonists (diltiazem and verapamil) may in reduce the vasoconstrictive effects and the degree of functional disturbance in animal models.[13–21] The loss of adrenergic balance due to a direct action of calcineurin on alpha receptors and the appearance of hypertrophy of the juxtaglomerular apparatus and other mechanisms that may produce a loss of renal vascular regulation causes by this drug.[13–22] The range of morphologic lesions is varied depending largely on the exposure time; vacuolization of the arterial tunica media (arteriolopathy), isometric vacuolization of the proximal tubule and hypertrophy of the juxtaglomerular apparatus are early lesions, whereas hypertrophy of the intima, mucoid deposits, glomerular hyalinosis and tubular atrophy with radial fibrosis have a later onset.[23] In the clinical context, nephrotoxicity caused by tacrolimus follows two distinct pattern which have different pathogenesis and prognosis[6]: early dysfunction (during the first 30 days) and late dysfunction (from the second month after the transplant onward.

The incidence of acute nephrotoxicity varies between 35% and 45%, but only 18% of patients develop acute renal insufficiency (creats < 3 mg/dL). Exposure to tacrolimus as the only nephrotoxic element is only present in 52% of cases with kidney dysfunction: in the other patients potentially damaging cofactors have they identified, including preexistent renal insufficiency, episodes of hypotension or hemorrhagic shock during surgery, nephrotoxic antibiotics and primary graft dysfunction. This subgroup requires more frequent hemodialysis (42% as compared to 79%) and displays of higher mortality rate (1.4% as compared with 45%).[6,7] Primary graft dysfunction (a result of preservation damage) is the predisposing factor that has received the most attention. Patients with normal hepatic function tend to not develop toxicity, whereas highly abnormal function provokes rapid deterioration, making it necessary to perform hemodialysis in up to 50% of cases despite reductions in drug doses. It has been emphasized that it is impossible to establish correlations between kidney function and biochemical indicators of liver damage (transaminases,

proteins, and clotting) except for LDH, total bilirubin, and cholinesterase. Correlation with total bilirubin may be explained as due to a damaging effect synergistic with tacrolimus. The reduction in cholinesterase expresses an alteration in liver synthesis capacity which is not identifiable with the usual parameters.[6–9] The presence of nephrotoxicity is also more marked during treatment for refractory rejection, namely patients with worse liver function and require higher doses of drug.10,14,15

In this study, we caused damage by ischemia and reperfusion of equivalent intensity in the three experimental groups, because the biochemical hepatic parameters reached similar maximum levels, but we encountered no significant differences. Nonetheless, only animals in group I (non–heart-beating donosr) were found to have creats >3 mg/dL, which represented a statistically significant difference from groups II and III (P = .035 versus I; P = .009 versus III). Although drug clearance (1.68 L/hr/kg) exceeded hepatic plasma flow (0.7 L/hr/kg), indicating the presence of a certain amount of extrahepatic clearance,[25] the drug is mainly metabolized in this organ.[26] Patients with poorer hepatic function maintain higher, or on occasions extremely high, blood concentrations of tacrolimus.[9] A correlation between high drug concentrations and the occurrence of toxic episodes has been proven,[8–26] but it is not essential to exceed the therapeutic range for nephrotoxicity to occur.[6,27,28] In two treatment groups (I and III), the blood concentration of tacrolimus remained within the therapeutic range (15.2 ± 2.4 compared with 12.3 ± 5.4 ng/mL; P = .4), even at the moment of death, and there were no significant differences between the two models.

In conclusion, ischemic liver damage did not seem to have been responsible for the nephrotoxicity caused by tacrolimus. The blood concentrations did not reach levels outside the therapeutic range, at least at the points we selected. The increased mortality of animals in group 1 was directly related to drug treatment, in view of the pathology findings, and is in contrast to the animals in the in situ ischemia model which, despite undergoing equivalent ischemic damage, had acceptable kidney function. The explanation for this fact may be found in differences in liver function that went undetected by the biochemical parameters used, and might be associated with faulty tacrolimus metabolization. The measurement techniques did not discriminate between native drug and nontoxic metabolites, the proportion of which may be different in the two models. Determination of the area beneath the curve,[29] with a larger number of sampling points, might have provided additional insights.

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Table 1. Plasma Level of Several Liver Function Parameters at 24 Hours After Reperfusion

Parameter/Group	I(LT)	II(IS)	III(IS-FK)	*P**
GOT (U/L)	1296 ± 867	1157 ± 465	1802 ± 1783	.676
GPT (U/L)	81 ± 27	122 ± 43	136 ± 133	.567
LDH (U/L)	2970 ± 985	2514 ± 1049	3299 ± 3646	.529
Prothrombine activity (%)	47.4 ± 15.0	73.5 ± 44.0	78.8 ± 19.7	.690
Albumin (g/dL)	1.62 ± 0.3	2.3 ± 1.2	2.5 ± 0.4	.67

Kruskal-Wallis test.

Fig 1. Evolution of creatinine plasma levels. Only the animals in group I reached concentrations in the range of acute renal failure.