Effects of Long-Term Administration of Recombinant Human Protein C in Xenografted Primates

Paolo Simioni,[1,9] Massimo Boldrin,[2] Sabrina Gavasso,[1] Michela Seveso,[2] Claudia Radu,[1] Cristiana Bulato,[1] Fiorella Calabrese,[3] Laura Cavicchioli,[4] Diana Bertini,[1] Giulia M. De Benedictis,[5] Federica Besenzon,[2] Nicola Baldan,[6] Luca Spiezia,[1] Mario Plebani,[7] Ermanno Ancona,[2,6] and Emanuele Cozzi[2,6,8]

Background. The role potential of recombinant human activated protein C (rhaPC), a recently developed molecule with anticoagulant and antiinflammatory properties, in prolonging survival in immunosuppressed primate recipients of porcine renal xenografts has been evaluated.

Methods. rhaPC was administered daily for 5 days (24 μg/kg/hr; group A; n=3) or throughout the postoperative period (8–24 μg/kg/hr; group B; n=2; or 24–48 μg/kg/hr; group C; n=4). Animals in group D (n=2) received rhaPC daily (24 μg/kg/hr) combined with recombinant human antithrombin (84 U/kg every 8 hr). Two animals served as control (group E).

Results. The results indicate that rhaPC is protective against fibrin deposition early after transplantation but does not prevent fibrin deposition and the occurrence of acute humoral xenograft rejection (AHXR) later on. Animals in the study survived between 8 and 55 days. At the dose used, rhaPC is able to prevent fibrin deposition in the graft in the first 2 weeks after xenotransplantation, except when it is administered in conjunction with antithrombin. However, rhaPC did not prevent the eventual occurrence of AHXR in primate recipients of porcine xenografts.

Conclusions. In this pig to primate model, rhaPC confers a short advantage in the prevention of early perioperative xenograft damage but does not represent an effective strategy for preventing AHXR.

Keywords: Recombinant activated protein C, Xenotransplantation, Coagulation, Humoral rejection, Primate.

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Genetically engineered donors pigs expressing human complement regulators or lacking expression of Galα1–3Galβ1–4GlcNAc-R structures (known as αGal epitopes) have considerably improved the survival of porcine xenografts transplanted in the primates (*1–4*). However, early graft failure primarily due to the onset of acute humoral xenograft rejection (AHXR) in the graft, generally associated with overt or nonovert coagulopathy, is observed in most cases (*5–7*).

To date, irrespective of the approach tested, fibrin deposition has been a consistent finding in xenografts explanted due to AHXR (*8–10*). Therefore, strategies aimed at preventing fibrin deposition (or favoring its removal) should prevent or delay the onset of this rejection process.

In this context, recombinant human activated protein C (rhaPC), a recently developed molecule with both anticoagulant and antiinflammatory properties, could provide beneficial effects to the graft. With regard to coagulation, rhaPC inactivates factor Va and factor VIIIa, two central factors in the clotting cascade. Beneficial properties of aPC that involve direct effects on cells require endothelial protein C receptors (EPCR) and protease-activated receptors (PAR-1) (*11*). These activities ultimately result in the downregulation of the proinflammatory and proapoptotic cascades and upregulation of antiinflammatory and antiapoptotic pathways. The antiinflammatory vascular properties of aPC can be divided

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[1] Department of Cardiologic, Thoracic and Vascular Sciences, 2nd Chair of Internal Medicine, University of Padua, Padua, Italy.
[2] CORIT (Consortium for Research on Organ Transplantation), Padua, Italy.
[3] Department of Pathological Anatomy, University of Padua, Padua, Italy.
[4] Department of Public Health, Comparative Pathology and Veterinary Hygiene, School of Veterinary Medicine, University of Padua, Padua, Italy.
[5] Department of Clinical Veterinary Sciences, University of Padua, Padua, Italy.
[6] Department of Surgical and Gastrointestinal Sciences, Clinica Chirurgica I, University of Padua, Padua, Italy.
[7] Department of Laboratory Medicine, Padua General Hospital, Padua, Italy.
[8] Direzione Sanitaria, Padua General Hospital, Padua, Italy.
[9] Address correspondence to: Paolo Simioni, M.D., Ph.D., Department of Cardiologic, Thoracic and Vascular Sciences, 2nd Chair of Internal Medicine, University of Padua, Via Giustiniani, 2, 35128 Padova, Italy.
E-mail: paolo.simioni@unipd.it

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into effects on endothelial cells and effects on leukocytes. aPC's effects on endothelial cells include inhibited release of inflammatory mediators and downregulation of adhesion molecules, thereby reducing leukocyte adhesion and infiltration, ultimately limiting tissue damage. rhaPC also has protective activities on the endothelial barrier functions mediated by EPCR and PAR-1 and consequent stimulation of sphingosine kinase 1 (*12*). Furthermore, aPC reduces cytokine release by leukocytes, chemotaxis, and migration. Collectively, these beneficial activities are referred to as rhaPC's cytoprotective activities (*13*). Interestingly, it has recently been shown that rhaPC possesses an Arg-Gly-Asp (RGD) sequence, which directly binds β_1 and β_3 integrins, therefore inhibiting neutrophil migration, both in vitro and in vivo (*14*). Thus, the binding of rhaPC to neutrophil integrins reduces neutrophil recruitment into tissues and may explain its protective effect toward sepsis.

At least three considerations underlie the use of aPC in this renal pig-to-primate xenotransplantation study. First, this study was aimed at exploring the capacity of aPC to stabilize the recipient clotting profile in this stringent transplantation model. Second, as aPC is able to prevent fibrin formation and deposition, we evaluated the capacity of this compound to prevent fibrin deposition in the graft and ultimately delay or prevent the onset of AHXR. Finally, we were interested in evaluating whether the antiinflammatory properties aPC could provide additional help in protecting against the damage mediated by the rejection process. Indeed, the interesting results on survival achieved in humans with severe sepsis (*15*), a clinical condition associated with profound discoagulopathy, and the evidence of both anticoagulant and inflammatory properties of rhaPC suggested to us that this molecule may have protective effects in our model.

Earlier studies from our laboratory in the pig-to-primate setting using recombinant antithrombin (rhAT), a molecule possessing both anticoagulant and antiinflammatory properties as does aPC, resulted in an improved coagulation profile but was not associated with significant prolongation of graft survival in immunosuppressed animals (*5*). Nonetheless, we speculated that, due to its central role in preventing fibrin deposition and its multiple antiinflammatory actions, aPC could protect the xenograft from AHXR, ultimately extending survival of xenografted primates.

The scope of this study was, therefore, to explore the potential of a long-term administration of the natural coagulation cascade inhibitor rhaPC in prolonging xenograft survival in immunosuppressed primate recipients.

MATERIALS AND METHODS

Animals

All experiments and procedures were conducted in accordance with the Italian Animals Act (Law No. 116 of the January 27, 1992) and were authorized by a special Decree of the Italian Ministry of Health. Thirteen ABO-matched large white/Landrace hDAF transgenic pigs (Imutran-Novartis) (*16*), 5 to 7 weeks of age and weighing between 5.5 and 8.1 kg, were used as kidney donors. Thirteen 4- to 5-year-old purpose-bred cynomolgus monkeys (*Macaca fascicularis*) from China and Philippines, weighing between 2.5 and 5.9 kg were used as recipients in the transplant studies. One additional cynomolgus monkey from China, weighing 3 kg, was used for the pharmacotolerance study.

Renal Xenotransplantation

Renal xenotransplantation has been performed as previously described (*17*). Each primate was given an immunosuppression consisting of up to four doses of cyclophosphamide intravenously perioperatively, cyclosporine A, steroids, and sodium mycophenolate. All xenotransplant recipients were also pretreated with GAS914 (Novartis Pharma AG, Basel, Switzerland), an injectable polymer expressing the carbohydrate moieties Galα1-3Galβ1-4GlcNAc-R, at a dose of 1 mg/kg subcutaneously on days -3, -2, -1, and on the day of transplantation (day 0) (*18*, *19*, *20*).

With aim of reducing the pretransplant hemolytic anti-pig antibodies titers, we developed an extracorporeal pig kidney perfusion (EPKP) model. Briefly, after a median laparotomy, sodium heparin (100 U/kg) was infused and left renal vessels clamped at their origin. The vessels were cannulated and the arterial and venous canulae were connected to one of the donor's kidneys for perfusion. After 2 hr EPKP, the perfused kidney was flushed with 10 mL saline to reduce blood sequestration. Considerable reduction of hemolytic anti-pig antibodies levels was achieved (data not shown) and the second, contralateral kidney was transplanted.

A total of 11 biopsies were performed in 10 animals between days 5 and 16, in accordance with the requirements of the local Animal Care and Use Committee.

Rejection was defined as an episode of deterioration in graft function (i.e., as an increase in serum creatinine concentration by at least 20% with or without oliguria) in the absence of any sign of technical causes of graft dysfunction ascertained by ultrasound. This was treated with a 3 to 5 day course of steroids (*1*).

Pharmacotolerance and Different rhaPC Regimens Applied to the Study

At the initiation of such studies, no data were available on the administration of rhaPC to immunosuppressed xenotransplanted primates. Therefore, rhaPC was administered long term (for 4 weeks) to a nontransplanted primate exposed to the same immunosuppression protocol used at our center in xenografted primates, to verify whether a long-term continuous treatment could result in adverse events (bleeding or other coagulation disorders) or interfere with drug levels.

To this end, we incannulated a cynomolgus monkey with a set up allowing continuous parenteral rhaPC infusion. The animal received the immunosuppressive protocol used to immunosuppress xenotransplanted primates and five cycles of 5 days each during which rhaPC (Xigris, kindly provided by Eli Lilly, Indianapolis, IN) was administered at the dose of 8 or 24 μg/kg per hr, alternatively. During the treatment, the animal underwent blood sampling daily for hematology and biochemistry. In addition, the coagulation parameters and the immunosuppressive drugs (cyclosporine A and mycophenolate) were measured three times a week. The drug dose was adjusted as needed.

Based on the excellent tolerability profile of such regimen, the xenografted animals in this study were divided into five treatment groups where rhaPC was administered at a dose comparable with or double that was used for the treatment of sepsis in humans (Fig. 1).

Primates from group A (n=3) received rhaPC on days 2 to 7 posttransplantation (24 μg/kg/hr). At the first signs of rejection, rhaPC treatment was reinitiated for 4 days at the same dose. In group B (n=2), rhaPC was administered on days 2 to 7 posttransplantation at the dose of 24 μg/kg per hr and, from day 8 onward, at 8 μg/kg per hr until graft deterioration. Episodes of rejection were treated with 4 days of rhaPC at 24 μg/kg per hr. Animals in group C (n=4) received 24 μg/kg per hr on day 1 and, thereafter, 48 μg/kg per hr for all posttransplant period. Animals in group D (n=2) received an anticoagulant drug combination composed of rhaPC (24 μg/kg/hr) and rhAT (84 U/kg, every 8 hr). Two animals that did not receive any anticoagulant were used as controls (group E). In all animals treated with rhaPC, the anticoagulation was discontinued in case of bleeding or marked reduction of platelet counts (platelets$<$100,000/mm^3).

Coagulation Studies

Blood samples were drawn and stored as previously described (*21*). Similarly, activated partial thromboplastin time (aPTT), PT fibrinogen, antithrombin,

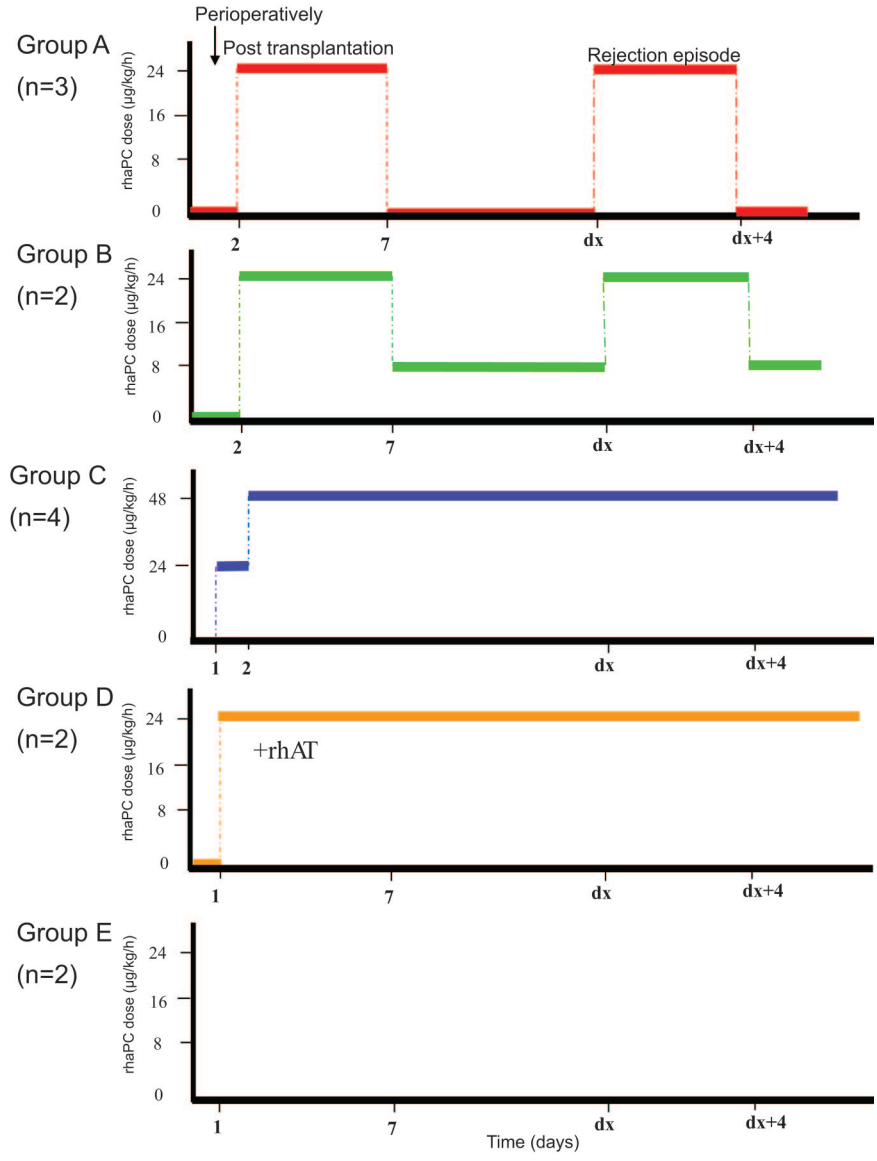

FIGURE 1. Schematic representation of recombinant human activated protein C (rhaPC) regimens in the five treatment groups. dx, the day of detection of a rejection episode.

protein C (PC) antigen, platelet counts, D-dimer (DD), and free protein S antigen and prothrombin $F_{1+2,}$ were determined as previously reported (*5*).

sEPCR

sEPCR was evaluated using EIA/ELISA for sEPCR (Asserachrom sEPCR, Diagnostica Stago Asnieres, France) according to manufacturer's instructions. Overt disseminated intravascular coagulation was diagnosed on the grounds of clinical and laboratory findings as previously reported (*21*, *22*).

Western Blot Analysis

rhaPC and PC purified from human plasma (Ceprotin, Baxter, Vienna, Austria) were diluted in sample buffer (6.25 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 100 mM DTT), heated for 3 min at 100°C and subjected to SDS-PAGE using 5% to 15% gradient gels under reducing and nonreducing conditions. rhaPC and PC were subsequently eletrotransferred to polyvinylidine difluoride membranes (Millipore, Milan, Italy) by semidry blotting. Membranes were incubated overnight at 4°C with blocking buffer containing 0.1% (w/v) albumin from chicken egg white (Sigma-Aldrich, Milan, Italy) in tris buffered saline tween (TBST) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and cut into strips. Each strip was incubated for 2 hr at room temperature with a 1:20 dilution in TBST of plasma from xenografted animals treated with rhaPC. Subsequently, strips were washed and incubated for 2 hr at room temperature with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (Sigma-Aldrich) diluted 1:500 in TBST. Finally, strips were washed with TBST and HRP activity was detected with 3.3′-DAB (Fluka, Milan, Italy).

Histopathology and Immunohistochemistry

Tissue sections from biopsy specimens taken during the postoperative period and from all explanted kidneys were stained and examined as reported elsewhere (*23*). A thorough macroscopic and histopathologic evaluation was also undertaken of the kidney, lung, liver, stomach, small intestine, large bowel, pancreas, heart, and bladder explanted at the time of euthanasia from each primate involved in the study.

RESULTS

Pharmacotolerance Testing in a Cynomolgus Monkey

The animal exposed to the pharmacotolerance test showed a slight weight loss during the observation period,

possibly related to the stress associated with the manipulation procedures and a loss of appetite due to the immunosuppressive drugs. Furthermore, a minor episode of spontaneous nasal bleeding and a minor muscular bleeding after an intramuscular injection, both of which resolved spontaneously, were observed during the rhaPC treatment.

PT, fibrinogen, platelets counts, hemoglobin, remained stable during the 4-week administrations of rhaPC. In contrast, a slight increase of aPTT above the normal range was observed during the administration period. At the end of the study, the animal was in a good general condition with normal blood counts. D-dimer was elevated before treatment, possibly due to the surgical positioning of the permanent infusion line and dropped progressively. F_{1+2} was stable within the normal range during the 4-week treatment.

Transplantation Studies

Animals in the study survived between 8 and 55 days (mean survival time, 24.23±13.66 days; median, 24 days). Recipient survival time, reasons for euthanasia and graft histology at autopsy are given in Table 1. Animals in group A had a mean survival time of 18.3± 6.6 days (range, 11–24 days; median, 20 days); animals in group B had a mean survival time of 23.5 days (range, 10–37 days); animals in group C had a mean survival time of 18±12.02 days (range, 7–32 days; median, 22.5 days); animals in group D had a mean survival time of 23±15.55 days (range, 12–34 days); animals in group E had a mean survival time of 41.5±19.09 (range, 28–55 days). The key clinical data recorded during the study are presented in Figure 2.

All xenografted animals had excellent initial graft function. In group A, two animals (1792 and 1496) underwent surgical repositioning of the stent by day 6, and one animal (6186) underwent surgery for hemoperitoneum. In all cases, euthanasia took place as a consequence of renal failure.

In group B, animal 1796 showed a progressive and irreversible graft failure that prompted the euthanasia on day 10. Animal 4114 showed excellent creatinine levels throughout most of its postoperative period, although spikes meeting the criteria for rejection episodes were observed and treated on postoperative days 4, 16, 26, and 36. Animal was euthanized on day 37 for animal welfare reasons.

In group C, animal 1598 underwent stent replacement on day 16 and had a stable creatinine until day 30. Subsequently, there was a progressive deterioration of graft function, associated with thrombocytopenia that prompted euthanasia on day 32. Animal 5596D was euthanized on day 8 for anuria due to ureteric obstruction and animal welfare reasons. Animal 7629C died of acute respiratory distress, on day 15, due to massive pleural effusion possibly secondary to fluid overload, during sedation for animal gavage. Animal 6923 had a progressive deterioration of graft function that led to euthanasia on day 30.

In group D, animal 8138A underwent stent replacement on day 6 and had a progressive deterioration of graft function that leads to euthanasia on day 12. Animal 5902C

TABLE 1. Survival, cause of euthanasia, and histologic findings

Group	Animal (group)	Survival (d)	Cause of euthanasia	Biopsy	Histology at euthanasia
A	1792	24	Renal failure/weight gain >20%	Day 6: IgM, C3 deposition, no fibrin, no C5b9	AHXR I, fibrin +
	1496	11	Renal failure/weight gain >20%	ND	AHXR II, fibrin +
	6186	20	Renal failure/respiratory failure	Day 16: IgM, IgG, C5b9, C3 deposition, no fibrin	AHXR II, fibrin +++
B	1796	10	Renal failure/ hypothermia	ND	Pyelonephritis+ureteral fibrosis, no fibrin
	4114	37	Weight gain >20%/ renal failure	Day 15: no IgM, IgG, C5b9, C3, no fibrin	AHXR II, fibrin +++
C	1598	32	Renal failure/welfare	Day 10: IgM, C5b9 deposition, C3 equivocal, fibrin equivocal	AHXR II, fibrin ++
				Day 16: C3 equivocal, fibrin equivocal	
	5596D	8	Surgical, ureteral obstruction	Day 5: ND	AHXR I, fibrin +
	7629C	15	Respiratory failure	Day 10: no IgM, IgG, C5b9, no fibrin	AHXR II, fibrin +
	6923B	30	Renal failure	Day 10: IgM, C5b9, C3 deposition, fibrin equivocal	AHXR II, fibrin +
D	8138A	12	Welfare/renal failure	Day 9: IgM deposition; C5b9, C3, IgG equivocal fibrin++, AHXR	AHXR II, fibrin ++
	5902C	34	Renal failure/welfare	Day 7: IgM, C5b9 deposition, no IgG, C3, fibrin+++, AHXR	AHXR II, fibrin +
E	2834K	28	Welfare/renal failure	Day 12: IgM, C3, C5b9 deposition, no IgG, no fibrin	AHXR II, fibrin +++
	6220B	55	Welfare	Day 7: IgM deposition, C5b9, IgG deposition, fibrin+, AHXR	AHXR II, fibrin ++

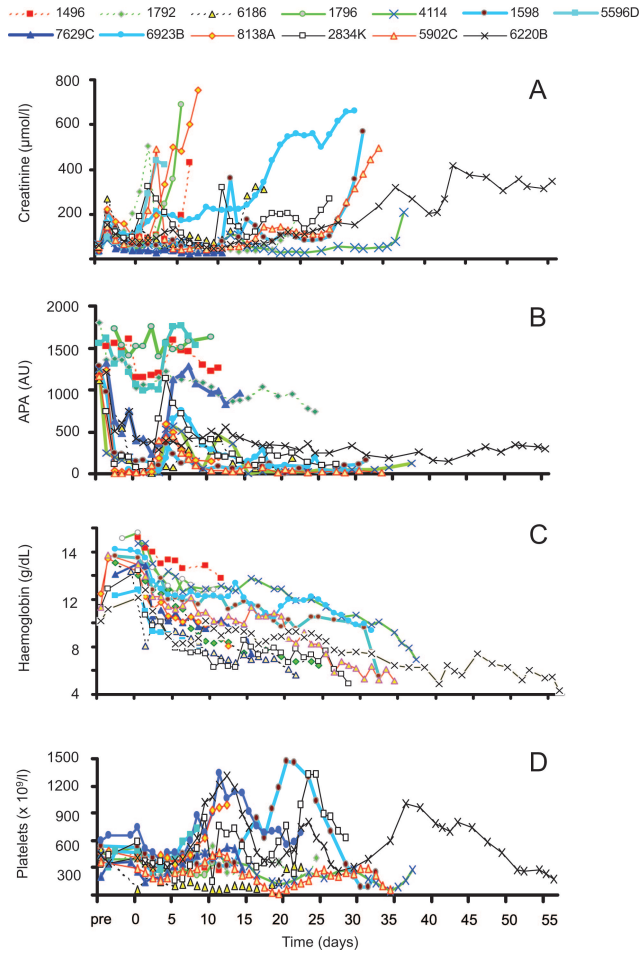

FIGURE 2. Daily levels of creatinine (A), hemolytic anti-pig antibody (B), hemoglobin (C), and platelet counts (D) in the 13 xenograft recipients.

underwent refashion of the ureteric anastomosis on day 7 followed by excellent graft function. At the beginning of the third week, the animal developed progressive thrombocytopenia (with nadir of 16,000 platelets/mm^3 on day 19), associated a leg hematoma. As precaution, rhaPC and rhAT were interrupted for 3 days and the animal was transfused. From day 28 onward, the animal showed progressive deterioration of graft function that lead to euthanasia on day 34.

In group E, animal 2834K had two episodes of graft rejection and underwent stent replacement on day 15. Subsequently, the animal showed deterioration of graft function and underwent euthanasia due to animal welfare reasons. Animal 6220B showed a fairly stable graft function (creatinine $<$140 Umol/L) until day 27 when it was treated for a deterioration of graft function compatible with rejection.

Twelve of the 13 animals included in the study were euthanized in the presence of kidney failure between 8 and 55 days after transplantation. In all these cases but one, a picture compatible with AHXR was seen in the explanted xenograft (Table 1).

A postoperative protocol biopsy was taken in 10 animals between days 6 and 16 and the histologic findings are summarized in Table 1. Fibrin was not observed or equivocal in the biopsy of all treated animals from groups A, B, or C (Fig. 3). However, in all cases, except animal 1796, fibrin was always observed in the explanted xenograft. It is of interest that fibrin deposition was less marked in the explanted xenografts animals from group C.

Some of the animals from groups A and B had an initially reduced intestinal motility in the first 2 weeks after transplantation possibly due to the prolonged surgical activity related to the session of EPKP. This was not observed in the latest animals due to the maximum care applied to preserve the temperature and humidity of the intestinal mass during EPKP.

At the doses used, rhaPC seems to be a safe drug even for prolonged treatment in primates. A far as anti-pig antibody titers, after transplantation all animals elicited at some stage anti-pig hemolytic antibody (Fig. 2B). Interestingly, in four cases neither the EPKP nor the grafts were able to abrogate the antibodies-mediated lytic activity of the sera. Further investigations conducted in our laboratory demonstrated that expression of the epitope recognized by the hemolytic antibodies was restricted to pig red blood cell and was not presented by porcine aortic endothelial cells (PAEC) (data not shown).

All animals in the study also showed a progressive reduction of hemoglobinemia, possibly related to the intense bleeding schedule in these primates in association with the administered immunosuppression (Fig. 2C).

Furthermore, a marked fluctuation in platelet count was observed in several animals (Fig. 2D). This was not associated with a specific rhaPC study group and was not dissimilar to that observed in previously published xenotransplantation studies in the primate.

Coagulation Tests

Coagulation tests performed failed to show significant differences between the four treatment groups and results were not significantly different from controls.

The levels of clotting inhibitors (AT, PC, and PS) and fibrinogen were consistent with the previously reported nonovert consumptive coagulopathy often observed after xenotransplantation (*4*, *20*). Figures 4 and 5 show the levels of PT, aPTT, fibrinogen, physiologic clotting inhibitors, and levels of EPCR, D-dimer, and F_{1+2}. The expected prolongation of aPTT was evident in animals receiving rhaPC. No relationship was observed between variations in these parameters and survival. In particular, sEPCR plasma levels were fairly stable throughout the postoperative course, regardless of the treatment administered. It is of interest that three of the four animals treated with the highest dose of rhaPC presented with the lowest sEPCR levels. However, these were already at the lowest degree of normality before transplantation. Furthermore, EPCR levels in treated animals did not seem to differ substantially from levels observed in control, untreated animals (Fig. 5). Similarly, the combination of rhaPC with AT after transplantation did not result in significant variations in the levels of sEPCR. As expected, D-dimer was considerably increased in all treated and untreated animals. In contrast, there was an unremarkable distribution in the levels of F_{1+2}, which seems to be increased in all animals after transplantation and was more pronounced toward the end.

As observed in other studies with xenografted monkeys (*5*, *21*), DD level increased rapidly immediately after transplantation and remained consistently high (up to 3500 ng/mL) throughout the follow-up, irrespective of rhaPC administration and clinical outcome (Fig. 5).

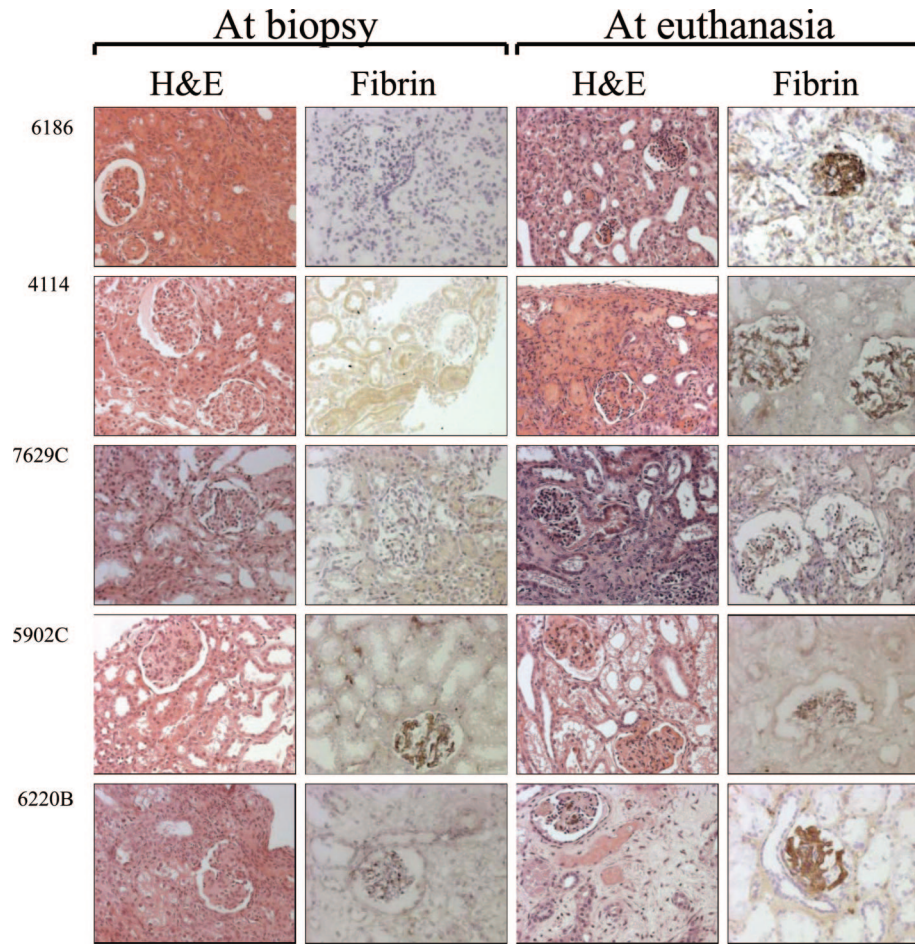

FIGURE 3. Hematoxylin-eosin and fibrin deposition at the time of biopsy (taken postoperatively, between days 6 and 16) and at euthanasia in a representative animal in each of the treatment groups.

Immunoblotting failed to show the presence of anti-protein C or anti-rhaPC in the plasma of xenografted animals drawn at the time of euthanasia.

DISCUSSION

Coagulation disorders represent a frequent cause of graft loss in large renal transplantation programs (*24*, *25*). In this context, rhaPC, a recently developed molecule, has been shown to have multiple protective effects that may improve the outcome of transplanted organs. In particular, aPC has been shown to prevent ischemia-reperfusion injury (*13*), to reduce the proinflammatory response and, ultimately, to control the coagulation responses (*15*). aPC also possesses important cytoprotective properties, including attenuation of endothelial cell injury after heatstroke in baboons (*26*) and reduction of apoptosis in allografted islets (*26*). In addition, in human sepsis, a condition where coagulation and inflammation need to be aggressively controlled, rhaPC has been shown to provide benefit and prolong patients' survival (*15*).

In the light of these encouraging results, we have tested the potential of such molecule in pig-to-primate xenotransplantation, a stringent preclinical model associated with massive activation of the coagulation and inflammatory cascades (*7*, *27–29*).

In this study, we have primarily evaluated whether rhaPC conferred any advantage as far as coagulation control, morbidity, organ, and recipients survival. In particular, we focused our attention on the ability of aPC to prevent thrombin generation and fibrin deposition in the xenograft, a well-established hallmark of AHXR (*28–30*).

At the initiation of such studies, no data were available on the long-term administration of rhaPC in the immunosuppressed primate. In this light, preliminary rhaPC tolerability studies were conducted in the context of our immunosuppressive regimen in the nontransplanted primate, based on rhaPC protocols resulting in improved survival in human sepsis. As the use of aPC is associated with risk of spontaneous bleeding, we developed four rhaPC regimens enabling us to fully explore the potential of this molecule, in terms of efficacy and safety, after long-term administration in xenografted primates. In one case, rhaPC was administered together with rhAT. Indeed, based on the evidence that both aPC and AT also present antiinflammatory activities, it was believed that such property could potentially counteract the detrimental effects mediated by inflammation in AHXR.

Overall, the data obtained in this study indicate that, regardless of the regimen used, rhaPC is able to prevent fibrin deposition in the graft in the first 2 weeks after transplantation. However, at the dose used, rhaPC did not prevent the eventual occurrence of AHXR in primate recipients of porcine xenografts.

To rule out that the loss of efficacy with time may be cased by due to an elicited anti-rhaPC antibody response, an immunoblotting assessment was performed using PC or

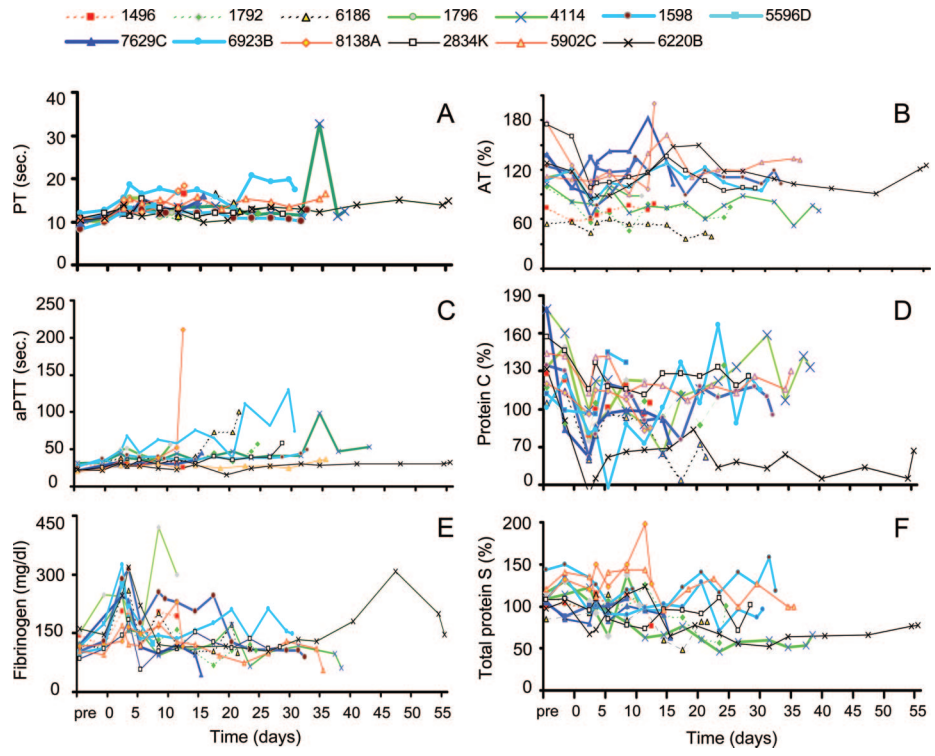

FIGURE 4. Daily levels of physiologic clotting inhibitors in the 13 xenograft recipients: prothrombin time (PT) (A), antithrombin antigen (AT) (B), activated partial thromboplastin time (aPTT) (C), protein C antigen (D), fibrinogen (E), and total protein S (F).

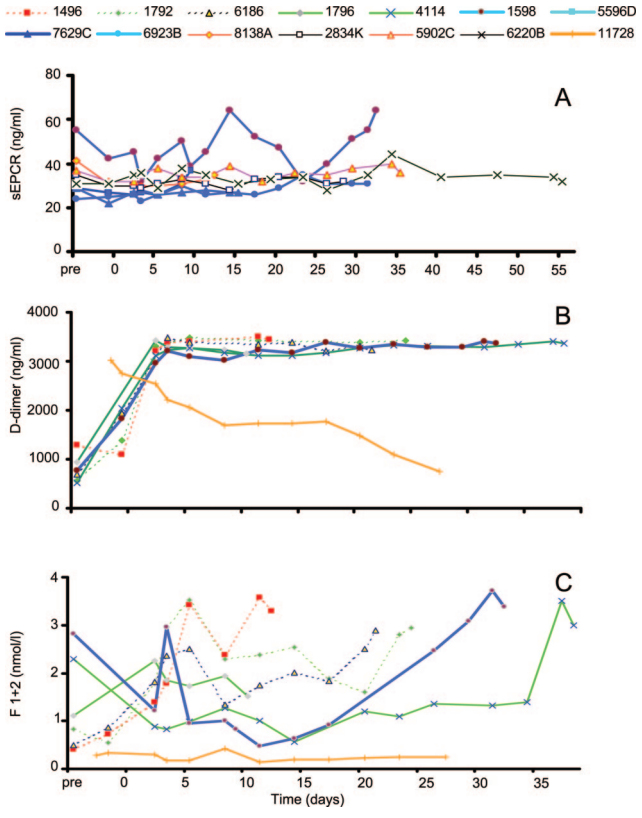

FIGURE 5. Daily levels of sEPCR (A), D-dimer (B), and F_{1+2} (C). Animal 11728 was used in pharmacotolerance studies only.

rhaPC. The data of our studies conclusively demonstrate that administration of rhaPC in immunosuppressed primates does not result in the development of anti-rhaPC antibodies.

Interestingly, in the biopsy specimens taken between 5 and 16 days after transplantation, fibrin deposition could not be observed or was equivocal in all animals treated with rhaPC only. This finding is in striking contrast with the observations in the animals treated with a combination of rhaPC and rhAT (group D) that showed a massive deposition of fibrin in the graft as early on as day 7. Interestingly, in a previous study, we could observe fibrin deposition in the graft in biopsy specimens taken on day 15 from animals treated with rhAT only in a similar xenotransplantation model (*5*).

Taken together, these results suggest that rhaPC seems to be protective against fibrin deposition early after transplantation, possibly due to both anticoagulant and antiinflammatory properties mediated by the compound. In this light, the detrimental effects observed after the administration of rhAT together with rhaPC is puzzling and remain to be elucidated. Indeed, we cannot rule out that the combined effect of the two drugs on thrombin activities may result in an imbalance between anticoagulant and procoagulant functions of thrombin and some impairment of inflammatory functions that resulted in excessive fibrin deposition.

When compared with the promising results obtained in humans with sepsis, the lack of efficacy of rhaPC in this primate xenotransplantation model is likely to be related to the profound differences in the coagulopathy presented by these two diverse conditions (*21*).

At euthanasia, histology showed marked fibrin deposition in the graft with a picture compatible with AHXR in all but one case. No remarkable differences could be noted between the treatment regimens used. Two hypotheses may

explain the apparent discrepancy between the absence of fibrin deposition in the early biopsies and its invariable presence in the explanted grafts. First, it is likely that the massive activation of the clotting cascade may, at the end, have exceeded the capacity of rhaPC to counteract fibrin formation and deposition in the graft. Alternatively, the actual local concentration of rhaPC may have been insufficient to prevent fibrin formation and deposition on the xenograft endothelial cells. In this view, the possibility of expressing physiologic or supraphysiologic levels of clotting inhibitors, in particular aPC, in the place where it is most needed (i.e., the xenograft) seems to offer considerable advantage over systemic administration of the drug.

Even though none of the animals developed overt disseminated intravascular coagulation, rhaPC did not prevent the compensated consumptive coagulopathy previously described by us (*21*) and others (*8*). rhaPC was not able to prevent the increase of D-dimer after transplantation. This finding may clash with those reported in humans treated with rhaPC for sepsis. However, one should note that in pig-to-primate xenotransplantation models, D-dimer levels always failed to be correlated with the intensity of the coagulopathy observed (*5*, *21*).

In conclusion, the short-term advantage conferred to the xenograft after rhaPC administration underline the potential of this agent as a protective adjunct to prevent early perioperative damage, such as that associated with ischemia-reperfusion injury or other inflammatory or thrombotic events. In this regard, rhaPC seems to be more effective than rhAT in preventing the onset of thrombin generation and fibrin deposition (*5*). On the other hand, survival in this study could not be improved suggesting that long-term administration of rhaPC does not represent an effective strategy for preventing AHXR.

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