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Ureteral Stenosis in HDAF Pig-to-Primate Renal Xenotransplantation: A Phenomenon Related to Immunological Events?

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The aim of this study was to analyze the incidence of ureteral stenosis in a life-supporting human decay-accelerating factor (hDAF) transgenic pig-to-cynomolgus monkey kidney transplantation model and determine the role of possible immunological events in its pathogenesis.

Thirty consecutive bi-nephrectomized cynomolgus monkeys received a kidney from hDAF transgenic pigs with or without a ureteral stent.

Four monkeys were euthanized prematurely after transplantation. In the remaining 26 cases, the mean survival was 24 ± 19 days. Except in one case, there was a close relationship between ureter and kidney in terms of type and severity of rejection. There were six ureteral stenoses; five were repaired by stent positioning and resurgery extended survival for an additional 16 ± 10 days. The stenotic ureters showed diffuse acute humoral xenograft rejection (AHXR), while all cases with no or only focal signs of ureteral rejection never revealed ureteral obstruction.

Use of a ureteral stent extends the survival of a xenografted primate, thereby helping to clarify the immunological events surrounding the onset of AHXR in kidneys in long-term xenograft recipients.

Key words: Kidney xenotransplantation, pig to primate, transgenic pig, ureteral complication

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Introduction

It is currently accepted that the problem of hyperacute rejection (HAR) in pig-to-primate renal xenotransplantation has been overcome by the introduction of pigs transgenic for proteins regulating complement activation, such as human decay-accelerating factor (hDAF) (1). However, for immunological reasons, xenotransplantation with organs from hDAF transgenic pigs (1) can only be performed in old-world nonhuman primates. In many cases, the cynomolgus monkey (*Macaca fascicularis*) is the preferred recipient species because it can be bred in captivity and is easy to handle. The Achilles' heel in pig-to-small-primate kidney transplantation surgery seems to be ureteroneocystostomy. In fact, an incidence of ureteral stenosis as high as 50% in long-term survivors has been reported by authors with great skill in the surgical technique of renal allotransplantation in cynomolgus monkeys (2). A similar but earlier incidence of urological complication was observed in our experience with the pig-to-cynomolgus xenotransplantation model, prompting an early suspension of the experiments. In our case, we speculated that an immunological reaction to the xenograft (which is far more vigorous in xeno- than in allotransplantation) might at least partially explain the high urological complication rate in our series.

The aim of this study was to investigate the incidence of ureteral stenosis in a renal hDAF pig-to-cynomolgus model and determine any association with immunological events, while also testing a new surgical solution for preventing the onset of renal failure secondary to urinary retention in order to enable a longitudinal study of AHXR in all of its phases.

Materials and Methods

Animals

Source animals were 30 ABO-matched piglets, transgenic for hDAF, produced by Imutran/Novartis (Basel, Switzerland) (3). The piglets were >21 days old and weighed 5.1–16 kg (mean 8.8 ± 2.8 kg).

Recipient primates were 30 purpose-bred cynomolgus monkeys imported from the Philippines (the first 17) or Mauritius (the last 13). Their weights ranged from 2.63 to 7.46 kg (mean 4.0 ± 1.2 kg).

All experiments were conducted according to the European Guide on the Care and Use of Laboratory Animals transposed by the Italian D.L.vo 116 of 27th January 1992, and were approved by the Italian Ministry of Health.

Surgical technique and immunosuppressive regimens

To minimize any learning curve effects, the surgical model (4) was applied first by two experienced microsurgeons, who handled all seven cases in group CYP and six of the first seven cases in group MTX, while the remainder of the operations were performed by a junior surgeon.

A renal life-supporting hDAF pig-to-cynomolgus monkey xenotransplantation model was used (4). At harvesting, ureters were not isolated before *in situ* organ perfusion to avoid vasospasm of ureteral vessels. Intraperitoneal organs were perfused and cooled with 350 mL of Celsior® (Imtix Sangstat Italia, Milan, Italy) at 4 °C and a pressure of 60 cm H_2O, and intraperitoneal crushed ice. The kidneys were harvested using the *en bloc* technique, paying attention to dissect the ureters with a large amount of peri-ureteral tissue to prevent damage to the ureter or its blood supply. The kidneys were subsequently divided at the back table. One kidney was transplanted and the other served as a control for histopathological and immunohistochemical studies. The right kidney was used in most cases (27/30). The mean weight of the transplanted kidneys was 27 ± 7 g (range 14–46 g).

Bilateral nephrectomy was performed in the recipient (cynomolgus monkey). The abdominal aorta and inferior vena cava were isolated and the renal vessels were anastomosed to them end-to-side. The mean cold ischemia time and time of anastomosis were 152 ± 34 min (range 79–232 min) and 43 ± 8 min (range 32–70 min), respectively.

As for the urinary tract, a uretero-cystoanastomosis was performed using an antireflux technique according to Leadbetter-Politano (in the first three cases) or the extravesical Lich-Gregoir technique (in the other 27 cases) (5). While we did not position any stent in the first nine consecutive cases, an Endo Sof double-J ureteral stent (Cook Urological Italia, Milan, Italy) was used in the remaining 21 to prevent ureteral stenosis. In detail, the stent was remodeled in water at 60 °C for approximately 20 min before use in order to partially straighten the pelvicalyceal end of the stent. After insertion of the ureteral portion, the remainder of the stent was placed in the bladder through a small hole in the vesical mucosa. Finally, ureteromucosal anastomosis and a tunnel with the bladder muscle layer were completed.

The transplanted animals received five different immunosuppressive regimens (Table 1; 6–9).

Post-transplant follow up

Post-transplant monitoring included clinical examination, hematological and biochemical evaluation, cyclosporine A and mycophenolic acid trough levels, hemolytic antipig antibody levels and blood and stool cultures. When graft function deteriorated, ultrasound was used to distinguish between organic and functional causes of graft dysfunction.

A diagnosis of ureteral stenosis was suspected on a clinical basis, evidenced by ultrasound and confirmed by surgical exploration (Figure 1) or necroscopy.

Termination of experiments and histopathological evaluation

Experiments were stopped on the basis of the animals' clinical condition (severe renal failure or systemic illness) or at the veterinary surgeon's recommendation.

All transplanted kidneys underwent histopathological and immunohistochemical examination as previously reported (1,6). The ureter of each transplanted kidney was extensively examined in serial sections distinguishing between the proximal, medial and distal parts to ensure that the findings were not limited to the areas with a less favorable blood supply. For histological and immunohistochemical investigations on the ureter we used the same techniques and antibodies as for the kidney graft analysis. Unimplanted pig kidneys and ureters were used as controls.

Results

We observed two cases of graft HAR on postoperative days 1 (C110) and 4 (Y634), one case of renal artery thrombosis on postoperative day 2 (Y034), and one case of anesthesiological complications prompting euthanasia at the time of surgery (C122) (Table 1). These four prematurely euthanized cases will not be discussed further. In the remaining 26 xenotransplanted animals, the mean survival was 24.4 ± 18.6 days (median: 20 days; range: 5–90 days).

Table 1 shows the histological findings in both kidney and ureter and the cases with ureteral stenosis in each xenografted primate. A close analysis of these data prompts several observations. First, ureteral AHXR was observed in 85% of cases (22/26) and ranged from 67% of cases in the CYP Group to 100% of cases in the GAS and ATIII Groups. In contrast, ACXR was observed in only one case (C118). Second, a close relationship was seen between ureter and kidney damage in terms of prevalence, type and severity of rejection in all cases but one (C118). Case C118 was the longest survivor (90 days) and the only animal in our series to present AHXR grade III in the kidney, in the absence of any humoral ureter damage (no IgG, complement or fibrin deposits), whereas ACXR grade I was observed in both the kidney and the ureter. Cold ischemia time and time of anastomosis in this animal were 155 and 35 min, respectively. Third, as far as rejection of the ureters is concerned, histological examination showed four different situations: no rejection of any type (C138, C130 and W922, Figure 2A); ACXR alone (C118, Figure 2B); focal AHXR (C106, C128, Y624, 869 and 222, Figure 2C); and diffuse AHXR (the remaining cases). Except for case C132, where a venous thrombosis was the cause of a diffuse fibrosis with ischemic necrosis, diffuse AHXR was represented according to two different patterns. The first, observed in animals C108, C136, C102, C114, 926, 071 and Y186, consisted of diffuse AHXR associated with wall thickening and a severe reduction of the lumen, particularly in the medial part. There were signs of diffuse mucosal erosion, hemorrhage, intraluminal blood vessel thrombosis (compatible with diffuse AHXR), fibrinoid necrosis, active fibroblastic proliferation with juvenile collagen deposits and mixed submucosal

Table 1: Immunosuppression therapy, histological findings, ureteral aspects and complications in the first 30 consecutive primates xenografted at our center

Immunosuppression	Case	Kidney		Ureter		Overall ureteral AHXR	Stent at surgery	Ureteral stenosis	Stenosis repaired with stent	Survival (days)
		AHXR	ACXR	AHXR	ACXR					
Group CYP										
I: CyP, CyA, Steroids										
M: CyA, MPS, Steroids	C106	II	–	focal	–	4 (67%)*	no	no	no	48
	C124	*II*	–	*diffuse+SMH*	–		*no*	*yes*	*14th p.o.d.*	*20*
	C108	*III*	–	*diffuse*	–		*no*	*yes*	*25th p.o.d.*	*40*
	C110§	HAR	–	–	–		no	no	no	1
	C118	III	I	–	I		no	no	no	90
	C128	I	I	focal	–		no	no	no	13
	C130	–	–	–	–		yes	no	no	5
Group MTX										
I: MTX, CyA, Steroids										
M: CyA, MPS/MTX, Steroids	C138	–	I	–	–	8 (89%)**	no	no	no	9
	C102	*III*	–	*diffuse*	–		*no*	*yes*	*2nd p.o.d.*	*9*
	C136	*III*	–	*diffuse*	–		*no*	*yes*	*no*	*6*
	C112	III	I	diffuse+SMH	–		yes	no	no	39
	C120	II	I	diffuse+SMH	–		yes	no	no	10
	C122§	–	–	–	–		yes	no	no	0
	C134	*III*	–	*diffuse+SMH*	–		*yes*	*yes*	*13th p.o.d.*	*34*
	A484	II	–	diffuse+SMH	–		yes	no	no	16
	C132	*III*	–	*diffuse*	–		*yes*	*yes*	*10th p.o.d.*	*41*
	C114	III	–	diffuse	–		yes	no	no	16
Group GAS										
I: CyP, GAS914, CyA, Steroids										
M: CyA, MPS, Steroids	Y624	II	–	focal	–	5 (100%)	yes	no	no	38
	Y913	III	–	diffuse+SMH	–		yes	no	no	21
	217	III	–	diffuse+SMH	–		yes	no	no	30
	926	II	–	diffuse	–		yes	no	no	16
	071	I	–	diffuse	–		yes	no	no	7
Group CO										
Donor treated pre-operatively with CO										
I: CyP, GAS914, CyA, Steroids										
M: CyA, MPS, Steroids	Y634§	HAR	–	–	–	3 (75%)***	yes	no	no	4
	W922	–	–	–	–		yes	no	no	5
	Y186	II	–	diffuse	–		yes	no	no	12
	Y034§	III	–	–	–		yes	no	no	2
	W946	III	–	diffuse+SMH	–		yes	no	no	27
	W918	II/III	–	diffuse+SMH	–		yes	no	no	37
Group ATIII										
I: CyP, GAS914, CyA, Steroids										
M: CyA, MPS, Steroids, AT III	869	II	–	focal	–	2 (100%)	yes	no	no	23
	222	II	–	focal	–		yes	no	no	23

Italic = cases with ureteral stenosis.

ACXR = acute cellular xenograft rejection; AHXR = acute humoral xenograft rejection; HAR = hyperacute rejection; SMH = smooth muscle hypertrophy; I = induction; M = maintenance; CyP = cyclophosphamide (Endoxan-Asta®, Asta Medica, Italy); CyA = cyclosporine A (Sandimmun Neoral®, Novartis Pharma AG, Basel, Switzerland); MPS = mycophenolate sodium (Myfortic®, Novartis Pharma AG, Basel, Switzerland); MTX = methotrexate (Methotrexate®, Wyeth Lederle, Aprilia, Italy); GAS914 = injectable polymeric form of αGAL (Novartis Pharma AG, Basel, Switzerland); induction steroids = methylprednisolone (Solu-Medrol®, Pharmacia & Upjohn, Milan, Italy); maintenance steroids = prednisone (Deltacortene®, Hoechst Marion Roussel, Scoppito, Italy); CO = carbon monoxide; AT III = antithrombin III (Genzyme Transgenic Corporation Biotherapeutics, Inc., Framingham, MA, USA)

§Animals not considered in the ureteral stenosis analysis owing to premature euthanasia: *one animal related to HAR; **one animal related to anesthesiological complications; and ***two animals related to HAR (one case) and arterial thrombosis (one case).

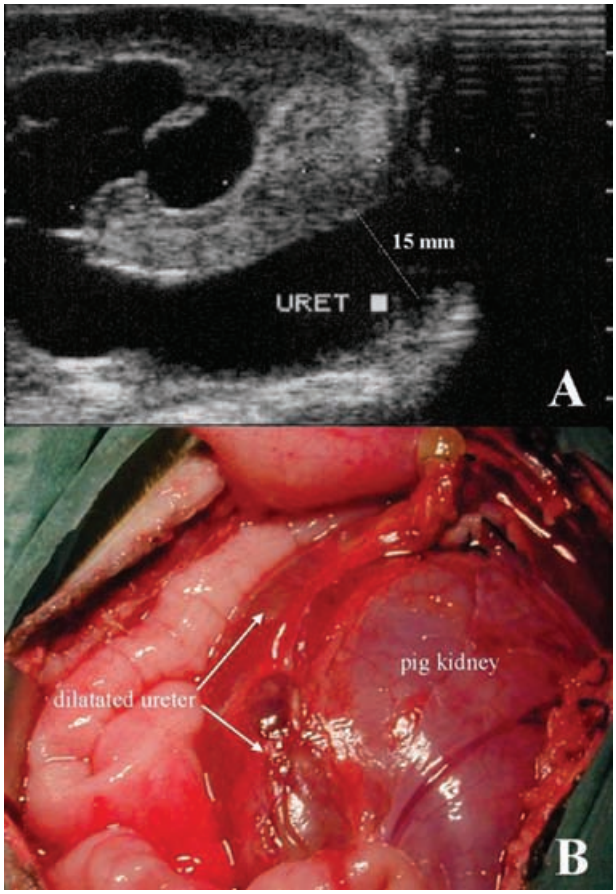

Figure 1: Ureteral stenosis was diagnosed by ultrasound and confirmed by surgical exploration. (A) Ultrasonography of obstructed ureter with pelvicalyceal and upper ureter dilatation. (B) Confirmation of ureteral dilatation by surgical exploration.

inflammatory infiltration (Figure 2D). Immunohistochemical examination revealed the presence of intravascular C5b9, C3, IgG, IgM and fibrin deposits on the wall of many small vessels of all stenotic ureters (Figures 2E–G). Three of these cases developed a ureteral stenosis, while positioning a stent at surgery prevented hydroureteronephrosis in the other four cases. The second pattern was characterized by diffuse AHXR associated with homogeneous ureteral wall thickening owing to smooth muscle hypertrophy. In all these cases, histological examination showed considerable smooth muscle hypertrophy of the external layer of the ureter with multifocal mucosal erosions, hemorrhage, vascular thrombosis and granulocyte infiltration (compatible with AHXR) in the mucosal and submucosal layers, associated with fibrinoid necrosis (Figure 2H). Immunohistochemical evaluation showed intravascular C5b9, C3, IgG, IgM and fibrin deposits on the wall of many small vessels in all these ureters. In all these cases, a ureteral

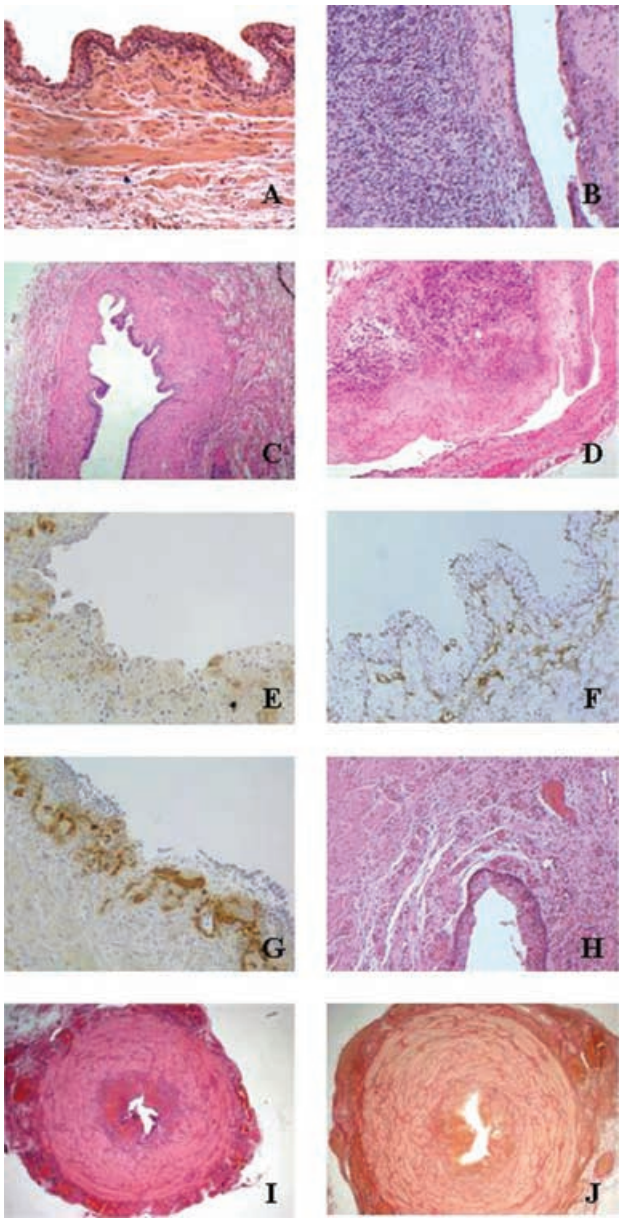

Figure 2: Histological patterns, immunohistochemical findings and ureteral aspects of transplanted ureters. (A) Normal aspects of the ureter in primate C138 (original magnification, ×2.5 and ×20). (B) Ureteral ACXR (C118, original magnification, ×25). (C) Focal ureteral AHXR (C106, original magnification, ×10). (D) Ureteral diffuse AHXR without smooth muscle hypertrophy (C108, original magnification, ×10). (E) Immunohistochemical examination showing C5b9, (F) IgM and (G) fibrin deposits (C108, original magnification, ×4). (H) Ureteral diffuse AHXR with smooth muscle hypertrophy (C112, original magnification, ×25). Transversal section of ureteral AHXR with smooth muscle hypertrophy, fibrinoid necrosis, diffuse mucosal erosion and intraluminal blood vessel thrombosis stained by (I) H&E and (J) elastic van Gieson (C112, original magnification, ×2.5).

stent was positioned at the time of transplantation or at resurgery owing to ureteral stenosis. Transversal section of the ureter demonstrated the critical role of the stent in maintaining ureter patency (Figures 2I,J).

As far as ureteral stenosis is concerned, this complication was observed in six cases in which diffuse AHXR was always detectable. Conversely, none of the cases with no or only focal signs of ureteral rejection revealed ureteral obstruction. In all but one case (C136) with stenosis, the stenosis was repaired by surgical *trans*-vesical positioning or re-positioning (cases C134 and C132) of a double pigtail ureteral stent. These re-operations were performed as soon as ureteral stenosis was diagnosed on postoperative days (p.o.d.) 2, 10, 13, 14 and 25 and enabled the transplanted animals' survival with no further stenotic episodes up to p.o.d. 9, 41, 34, 20 and 40, respectively. In primate C130, stenosis was associated with necrotizing enteritis, which prevented surgical repair and the animal was euthanized on p.o.d. 6. Considering all reoperated animals, resurgery extended survival by 16 ± 10 days (range 6–31 days).

Discussion

The development of pig-to-primate renal transplantation models is a critical step in the evaluation of organs derived from genetically engineered donor pigs. In this context, particular attention must be paid when testing transgenic animals expressing species-restricted molecules such as the human complement regulators (i.e. hDAF, CD59 or CD46). As these molecules are more effective in some primate species, data generated by Tucker have convincingly demonstrated that cynomolgus monkeys are the most suitable purpose-bred species available for these studies (10). On the other hand, their small size gives rise to a high rate of surgical (and particularly urological) complications. Even surgeons skilled in renal allotransplantation in small non-human primates have reported a high incidence of ureteral stenosis (2). As far as pig-to-primate kidney xenotransplantation models are concerned, a high incidence of hyperacute or humoral rejection prevents long-term survival and this may have led to an underestimation of the potential prevalence of urological complications. Kozlowski nonetheless reported two ureteral stenoses in a series of five primate recipients of a porcine renal xenograft with a median survival of 9 days (11). In another series, Sablinski recorded only one urinary complication in 16 cases, but the median survival in his group was only 5 days (12). Similarly, Zaidi reported no urological complications in his seven hDAF renal xenotransplanted primates with a median survival of 13 days (13). In other reports concerned primarily with studying immunological processes, the exclusion of animals as a result of surgical events may not have been reported in detail.

In this study, we evaluated the first 30 consecutive primates operated at our center whose median survival was 20 days, with eight animals surviving for 34 days or more. The overall ureter complication rate in this series was 23%.

Acute rejection is a major cause of ureteral stenosis both in experimental xenotransplantation (11,14) and in clinical allotransplantation (15,16). This process equally affects the kidney and ureter, and the resulting edema, with or without ischemia, may lead *per se* to ureter obstruction. The sequel of vascular damage secondary to rejection includes fibrosis and this may ultimately result in ureteral stenosis. Our data show that 5/6 animals with ureteral obstruction had at least one rejection episode postoperatively. By contrast, no ureteral stenosis was recorded among the animals with no, or only focal, ureteral AHXR at postmortem.

The immunosuppressive regimens used in this study did not appear to influence the prevalence of ureteral rejection, though the number of animals in each treatment group was too small for a conclusive evaluation.

It is noteworthy that ureteral rejection was seen in 85% of cases in our xenotransplantation model, and it was diffuse in 77% of cases. Kozlowski et al. were the first to describe in detail this complication when pig organs are transplanted into primates (11). Similarly, Buhler et al. (14) observed AHXR in the ureter in a pig-to-baboon kidney transplantation model, but when hDAF pig kidneys were used they found no ureteral AHXR at graft excision on days 28 and 29. At least four considerations may explain the differences between our results and Buhler's. First, Buhler's donor pigs were considerably larger (22–35 kg) than ours: we substantially share Buhler's view that small ureters (possibly with a less developed vascularization), such as those of 4–8 week-old pigs, may facilitate early venous occlusion, which is an early feature of AHXR (17). Second, substantially different immunosuppressive approaches were used in the two studies. Third, we used cynomolgus monkey as the recipient species, whereas Buhler used baboons. Finally, the ischemia time may have differed between the two series.

In addition to acute rejection, other causes may explain the development of ureteral stenosis. Unquestionably, the small size of the swine ureters used in our experiments could represent a cofactor responsible for the high rate of urological complications. This is supported by the high incidence of complications reported in pediatric kidney transplantation where ureteral obstruction ranges from 0.9 to 29.4% (18,19). Two different types of ureteral stenosis were documented in these reports: one consisted of a short stenosis at the ureterovesical anastomosis level, while a longer ureteral stenosis or local tissue fibrosis caused urine retention in the other (19). In the first case, the anastomotic technique was the main factor influencing the onset of stenosis. Some authors reported excellent results with the intravesical (Politano-Leadbetter) technique (20), so we initially used this method for the xenotransplantation program at our center. We performed

no uretero-ureterostomy because it is usually considered a second choice in kidney transplantation (21) and its use is discouraged in the case of native homolateral nephrectomy (22). Results were disappointing in our first three transplants, however, as two cases were associated with ureteral stenosis. Though other factors (e.g. ureteral rejection) were recognized, we consequently switched to the Lich-Gregoir ureterocystostomy technique (23,24), but two of the next five cases nonetheless developed early stenosis. In the light of these unsatisfactory observations, we decided to adopt a ureteral stent. The chosen stent can be left in place for more than 6 months with no reported untoward effects (25) and is similar to the type used in pediatric kidney transplantation, which reportedly reduces urological complication rates (26). Using a similar approach, Eckhoff was able to substantially reduce the incidence of urological complications in a series of 262 ureterovesical anastomoses in small nonhuman primates (2).

We observed no short ureteral stenoses of the cystoureteral anastomosis in our pig-to-primate renal xenotransplantation model, but only long ureteral stenoses. In addition to acute rejection, ureteral ischemia resulting from poor vascularization due to an insufficient arterial supply to the ureter (27) or venous occlusion (28) is one of the most frequent causes of extended stenosis, meaning that meticulous care during harvesting and transplantation is crucial to preventing the onset of ureteral complications after renal allotransplantation and possibly when undertaking pig-to-primate xenotransplantation (29). Finally, an arteriolopathy in the kidney (possibly also involving the transplanted ureter) related to the use of cyclosporine (30) cannot be ruled out as a possible cause of ureteral ischemia and consequent long stenosis in this model.

Several elements would suggest, however, that the long segment ureteral stenosis observed in our model is related more to AHXR than to ischemic insult. First, apart from the one case of venous thrombosis, full-thickness necrosis or fibrosis of the ureter was never observed in our study. Second, the histological damage consisted in findings compatible with AHXR and was located exclusively in the mucosal and submucosal layers. There was hypertrophy of the smooth muscle layer, however, which is substantially different from the picture one would expect in the case of ischemic damage. Third, immunohistochemistry was indicative of AHXR (1). Finally, the assumption that ureteral stenosis of the long segment variety may result from rejection is also supported by several other observations both in experimental (11,14) and clinical renal transplantation (15,16). Ischemia cannot be definitively ruled out, however, particularly because it may occur as a result of the vascular damage observed in AHXR.

Using a stent at the time of transplantation prevented the onset of hydroureteronephrosis and modified the macroscopic pattern of ureteral rejection in all cases surviving more than 16 days postoperatively. Indeed, stenting enabled active fibroblastic proliferation and juvenile collagen deposition in the immunologically damaged ureters, in association with smooth muscle hypertrophy. Progression of the fibrosis and smooth muscle hypertrophy ultimately led to total involvement of the ureteral wall with homogeneous wall thickening instead of the short median ureteral stenosis observed in cases where a stent was not used.

Given that not positioning a stent at the time of transplantation resulted in a 50% ureteral complication rate (stenosis), the substantially extended survival achieved in the stented recipients offers at least two considerable advantages. First, having eliminated one of the most frequent causes of death, we can focus on studying the AHXR process in renal xenotransplants to identify early indicators of xenograft rejection or possible targets of novel immunosuppressive strategies. Second, preventing ureteral complications enables us to further explore such new immunosuppressive approaches, their mechanisms of action and their long-term complications, ultimately reducing the overall number of animals needed.

Conclusions

Life-supporting hDAF pig-to-cynomolgus monkey renal xenotransplantation is a good model for xenotransplantation study, but it is burdened by a high urological complication rate, mostly involving ureteral stenosis attributable to immunological, technical or anatomical reasons. While the technical and anatomical problems can be overcome by a careful surgical approach during harvesting and transplantation, AHXR affecting the kidney and ureter can lead to early ureteral obstruction and prompt the premature interruption of the experiment. In our model, the prophylactic use of a ureteral stent extends the xenografted primate's survival and enables better animal handling during the AHXR process, so that more light can be cast on the sequence of immunological events surrounding the onset of AHXR in a kidney in long-term xenograft recipients.

In the light of our experience in this delicate and ethically demanding model, we recommend the routine use of ureteral stents when renal xenografts are transplanted into small primates.

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