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ORIGINAL ARTICLE

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Analysis of pig-to-human porcine endogenous retrovirus transmission in a triple-species kidney xenotransplantation model

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Abstract Clinical pig-to-human xenotransplantation might be associated with the risk of transmission of xenozoonoses, especially porcine endogenous retroviruses (PERVs). We have established a pig-to-humanised-cynomolgus monkey xenotransplantation model allowing the analysis of potential PERV-transmission from normal or transgenic porcine organs to human vascular tissue. Pig-to-human kidney xenotransplantation was performed in cynomolgus monkeys. An interposition graft constructed from a human saphena vein replaced the porcine kidney vein. After graft rejection and/or death of the recipient (survival 2, 4, 6, 13, 16, 19 days), the human interposition grafts were removed. Human endothelial cells (huECs) were isolated from the interposition grafts and cultivated in vitro. Explanted human vascular tissue, isolated huECs, plasma and serum samples of the graft recipients were characterised by flow cytometry and immunohistochemistry and screened for indications of PERV transmission by quantitative polymerase chain reaction (PCR), reverse transcriptase–polymerase chain reaction (RT–PCR) and RT assay. PERV-specific immune response of recipients was analysed by Western blot. No evidence of PERV infection or PERV-specific immune response was detected.

Keywords Xenotransplantation · Pig endogenous retrovirus · PERV

Introduction

Pigs probably represent the most suitable donor species for xenotransplantation purposes. Hyperacute rejection of porcine organs by primate recipients has been overcome by the introduction of pigs transgenic for human complement regulators. Currently, other problems, including later xenograft rejection mechanisms, problems of xenophysiology and the risk of transmission of zoonoses, have moved into the focus of xenotransplantation research. The risk of transmission of pig pathogens, especially the transmission of porcine endogenous retroviruses (PERVs), led to controversial discussions among clinicians and scientists.

Although the existence of these viruses in pigs has been known for many years [1], only the finding of in vitro infection of human cells [2] demonstrated the relevance of PERV for xenotransplantation. Meanwhile, it has become likely that all pig strains and breeds contain PERV elements [3, 4, 5, 6, 7, 8] and that the vast majority (but not all) pig strains express functional and humanotropic PERV. PERVs are released by a variety of primary pig cells, including peripheral blood mononuclear cells (PBMCs) [8] and endothelial cells [5], and there is evidence for the existence of PERV particles in porcine serum [4, 9].

Recent investigations of patients after limited contact with porcine cells or tissues did not provide any evidence for PERV infection (e.g. [10] for review see [11]). The patient samples investigated are the most suitable that are currently available, and the results are encouraging. These studies, however, do not closely mirror clinical xenotransplantation, as current immunosuppressive strategies, which are believed to impair PERV virolysis, such as the use of complement regulator transgenic donor organs [12], antibody absorption, fluid phase complement inhibition and heavy conventional immunosuppression, have not been applied.

Cells of several small animal species, including mouse, rat, cat and mink, have been demonstrated to express PERV receptors and, in some instances, to be susceptible to PERV infection [2, 8, 13, 14]. Because of this, small animal models are candidates as a means to compare factors that might affect PERV transmission in vivo. In contrast, it is noteworthy that all small animal models proposed to date suffer from the fact that possibly the most important human barrier against PERV infection, Gal-α1,3-Gal mediated virolysis, does not exist in these species [2, 8, 13, 14, 15]. Moreover, as immunosuppressive protocols comparable to those in primates have not yet been applied in mice, a close imitation of clinical xenotransplantation models may remain difficult to achieve.

It can be argued that PERV infection issues might be best addressed by use of non-human primate infection models. Several studies have supplied evidence for PERV infection of certain non-human primate cells of different species, including gorilla, chimpanzee, baboon and rhesus monkey [13, 16, 17]. In contrast to the in vitro studies mentioned above, no evidence for PERV infection has been detected in the recent in vivo infection studies using non-human primates [18, 19, 20, 21]. However, the value of non-human primate models for xenotransplantation risk assessment is controversial, as all non-human primate cells tested so far seem to be less permissive than certain human cell lines for PERV infection [19, 20, 21].

To combine the advantages of the human system with those of non-human primate models, we have developed a "humanised" pig-to-cynomolgus-monkey xenotransplantation model (Fig. 1). Unmodified as well as human complement regulator (CD59) transgenic kidneys have been transplanted into cynomolgus monkeys. In addition, we replaced the donor kidney vein with a human interposition graft constructed from a patient's vena saphena (Fig. 2). An infectious virus released by the porcine kidney would pass the human interposition graft immediately, thereby achieving close contact with human endothelial cells (huECs), which have been shown to bear PERV-specific receptors and to be highly susceptible to productive PERV infection [6].

Following kidney transplantation, profound pharmacological immunosuppression, supplemented by the continuous administration of a CI inhibitor, was given to the recipients. These strategies, which might enable survival of PERV particles in the recipients' serum, mirror clinical xenotransplantation settings and allowed for analysis of potential PERV in vivo transfer to huECs.

Material and methods

Animals

We used six cynomolgus monkeys (*Macaca fascicularis*) weighing between 3.3 kg and 4.7 kg, between 4.5 and 5 years of age, as models. The animals were purchased from the German Primate Centre, Göttingen. The recipients were immunosuppressed with a combination therapy of cyclophosphamide (CyP) induction, cyclosporin A (CyA), low-dose steroids, mycophenolic acid,

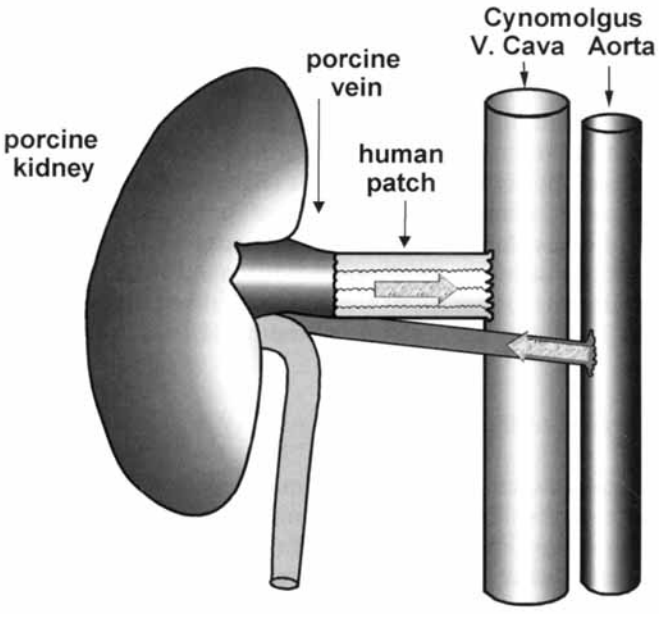

Fig. 1 Topographic scheme of the porcine and the human graft in situ. *Arrows* indicate direction of the blood flow

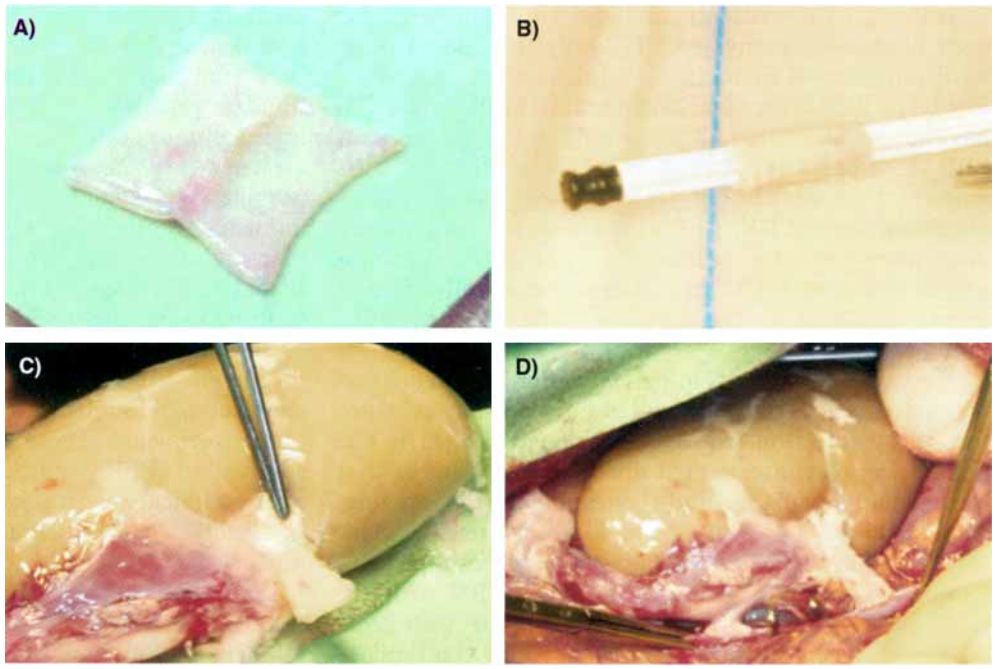

Fig. 2A–D Construction and transplantation of the human interposition graft. **A, B** Construction of the vascular graft from two human vena saphena fragments. **C** Anastomosis of the vascular graft to the porcine kidney vein (following resection of the distal part of the porcine kidney vein). **D** Porcine and human graft in situ

and a short course of a supplemental C1-inhibitor (C1-Inh) [22, 23].

Three unmodified and three transgenic large white landrace pigs were used as donor animals. The pigs were between 8 weeks and 18 weeks old and weighed between 18 kg and 23 kg. The unmodified pigs were obtained from Schweinezuchtverband Weser-Ems, Oldenburg, Germany. The transgenic pigs were produced by microinjection of a mini-gene construct that coded for the human complement-regulating protein CD59 [24] and were obtained from the FAL Mariensee, Germany.

During all animal experiments the *Principles of Laboratory Animal Care* (NIH publication no. 86-23, revised 1985) were followed, as well as the specific German law on the protection of animals. All experiments were approved by the local authorities (Bezirksregierung, Hanover) for animal welfare. Donors (patients undergoing cardiovascular revascularisation surgery) of human saphena vein segments gave written informed consent before their operation; the procedure was approved by the local ethics committee of the MHH.

Surgical technique

Non-transgenic and hCD59 transgenic porcine kidneys were transplanted into cynomolgus monkeys. A "life supporting" situation was created by ligating the recipient native ureters [25]. We replaced the porcine vena renalis by an interposition graft constructed from human vena saphena (Fig. 2). After rejection of the porcine kidneys or death of the recipients, the human interposition grafts were removed and the huECs were isolated and analysed for detection of potential PERV infection. Moreover, blood, serum and tissue samples of the transplanted animals were collected at different time points.

Preparation of recipient's peripheral blood leukocytes

Cynomolgus peripheral blood leukocytes (PBLs) were prepared from EDTA-blood by centrifugation at 1,800 *g* for 10 min at 4°C. Erythrocytes were lysed with 10 volumes of 0.2% NaCl for 1 min followed by the addition of the same volume of 1.6% NaCl. After washing the cells with PBS for 5 min at 450 *g*, we lysed 1×10^6 PBLs in 100 µl of 200 µg/ml proteinase K.

Plasma samples and control sera

Plasma was prepared from EDTA-blood samples by centrifugation at 3,400 *g* for 10 min at 20°C and was stored at −70°C until required for further use. Serum was retrieved by centrifugation of whole blood for 10 min at 600 *g*.

Isolation and culture of cells

Endothelial cells from the explanted grafts and control veins from recipients and donors were isolated by incubation of the inner layer of the grafts or control

veins with 0.1% collagenase type A in PBS for 10 min to 30 min at 37°C. After rinsing the detached cells with Earles M199/20% FCS, we resuspended the cells in 2 ml endothelial cell basal medium with supplements (PromoCell, Heidelberg, Germany). After two to eight passages, the cells were characterised by flow cytometry. Cell lysates, RNA and culture supernatant of the cells were prepared for further analysis as described below.

Flow cytometry analysis

Cells were harvested with trypsin-EDTA (PAA Laboratories, Linz, Austria). After being washed twice in PBS, cells (50 µl of 2×10^6 cells/ml in PBS) were incubated with 50 µl monoclonal antibody anti-human CD31 (clone JC70A; Dako, Glostrup, Denmark), anti-human CD31 (clone WM59; Pharmingen, San Diego, USA), anti-human CD90 specific for fibroblasts (clone AS02; Dianova, Hamburg, Germany) or 50 µl biotinylated Griffonia simplicifolia 1 isolectin IB4 (Vector Laboratories, Burlingame, USA) in microtitre plates at 4°C for 1 h. Mouse IgG1 (Dako) was used as isotype-matched control. Cells were washed three times in PBS and incubated for 30 min at 4°C with 50 µl FITC-conjugated goat anti-mouse IgG (Becton Dickinson, San Jose, USA). Binding of the lectin was visualised with streptavidin–phycoerythrin (Vector Laboratories). After cells had been washed twice in PBS, the fluorescence was analysed with a FACSCalibur (Becton Dickinson) equipped with CellQuest software.

Immunohistochemistry

Snap-frozen pieces of the veins were embedded into a Tissue Tek medium (O.C.T. compound; Sakura, Netherlands) and processed. Frozen tissue samples were cut with a cryostat, air dried on slides and stored at −80°C until required for further analysis. A standard two-step indirect staining technique, using mouse anti-human CD31 (clone JC70; Dako) that tested as not cross-reacting with cynomolgus CD31 ([26], our observation) and biotinylated horse anti-mouse IgG secondary antibody (Dako), was performed. Streptavidin peroxidase and diaminobenzidine (Dako) served as detection system.For counterstaining we used Härris-Hämatoxylin (Merck, Darmstadt, Germany). Porcine, human and monkey veins were used as controls.

Polymerase chain reaction specific for PERV and pig DNA

One million cells (endothelial cells and cynomolgus PBLs) or, alternatively, five slices of the tissue samples (20 µg) were lysed in 100 µl of 200 µg/ml proteinase K in polymerase chain reaction (PCR) buffer for 3 h at 56°C, followed by 10 min inactivation at 95°C. Of these crude extracts, 3.5 µl served as a template for the PCRs, which were performed with PERV pol-specific primers essentially as described previously [5, 6]. A second primer pair (5′-caaccagttgagttcctggttgata-3′; 5′-gtactgtctgactgatactggtgt-3′) specific for PERV pol was applied using the same cycling parameters. Pig-specific PCR was performed with cytochrome oxidase II (CyOII) [28] or pig centromere-specific primers (5′-TAGCCATGCTGCATGTAATGC-3′; 5′-GGAGCGTGGCCCAAT-3′). β-actin-specific primers (5′-GACGAGGCCCAGAGCAAGAG-3′; 5′-ATCTCCTTCTGCATCCTGTC-3′) were used for internal positive controls. PCR sensitivity was determined using porcine PK15 cells: different quantities of lysed PK15 cells were mixed with lysed human PBLs. The sensitivity of the applied primer pairs was one PK15 cell in a background of 10^5–10^6 human cells for PERV pol. Pig-specific primers (CyOII and centromere) allowed the detection of one PK15 cell in a background of 10^6–10^7 human cells.

All samples were tested at least three times, and those that yielded unclear results were retested by means of real-time PCR [10]. DNA for real-time PCR analysis was prepared from tissue samples with a QIAamp kit (Qiagen, Hilden) in accordance with the manufacturer's instructions. Primers and probes specific for swine leukocyte antigen (SLA) and PERV pol were used. (SLA: 5′-GCCCTGGGCTTCTACCCTAA-3′; 5′-TCTCAGGGTGAGTGGCT CCT-3′; 5′-FAM-CCAGGACCAGAGCCAGGACATGGAGCTCGT-TAMRA-3′ and PERV pol: 5′-AGCTCCGGGAGGCCTACTC-3′; 5′-ACAGCCGTTGGTGTGGTCA-3′; 5′-FAM-CCACCGTGCAGGAAACCTCGAGACT-TAMRA-3′). Real-time PCR reactions were performed on 100 ng DNA in a 25 µl reaction mix (QuantiTect Probe PCR Kit; Qiagen) containing 12.5 µmol of sense and antisense primers. The reactions were cycled at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. All reactions were quantified against known copy-number plasmid controls. Detection limits are 10 to 30 copies using plasmid controls. For internal positive control, DNA, isolated from the remaining kidneys of the donor pigs, was used.

Detection of PERV-RNA in plasma samples

RNA from plasma samples was prepared using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions. For internal positive control, RNA was purified in parallel from the same plasma samples but spiked with 10^6 molecules of a 995 bp PERV pol RNA fragment. This PERV pol RNA had been generated by in vitro transcription from a plasmid

containing a slightly modified PERV pol sequence. This artificial PERV pol sequence was similar to the corresponding genomic sequence, despite an internal stretch of 17 bp with modified sequence but unchanged base content. The modified internal sequence allowed us to control whether amplification within the spiked samples was due to the artificial PERV RNA or due to natural genomic or messenger PERV RNA. cDNA synthesis and subsequent PCR have been described earlier [6]. The detection limit was 10^3 RNA molecules per 125 µl serum.

Reverse transcriptase–PCR-based assay for reverse transferase activity

Measurement of reverse transcriptase (RT) activity in the cell culture supernatant was performed as described earlier [6]. The detection limit of the assay was 10^{-7} U AMV reverse transcriptase per sample.

Western blot assays

Western blot assays were performed as previously described [29] with slight modifications. A high-titre human cell adapted PERV (PERV/5°) [30], produced by infected human 293 cells, was purified by standard ultracentrifugation methods using sucrose gradients. Gradient fractions with peak RT activity were pooled and used for denaturing tricine-based polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membrane (Millipore, Bedford, Mass., USA) by electroblotting. Membranes were blocked using 3% bovine serum albumin in tris-aminomethane buffered saline. For detection of IgM or IgG, blots were incubated with 1:100 dilutions of the sera to be tested, followed by incubation with a peroxidase-coupled anti-human IgM antibody (Sigma, Deisenhofen, Germany) at 1:1,000 or, alternatively, by a 1:5,000 dilution of peroxidase-coupled protein G (Biorad, Hercules, Calif., USA). Antibody binding was visualised using metal-enhanced diaminobenzidine and peroxide (Pierce, Rockford, Ill., USA).

Measurement of RT activity in serum samples

For detection of RT activity, a commercial high-sensitivity assay (Cavidi Tech, Uppsala, Sweden) was used according to the manufacturer's instructions. The linear measurement range of the assay was 0.04–300 µU of recombinant Mn^{2+}-dependent reverse transcriptase per well. The detection limit of 0.04 µU corresponded to the presence of 0.8 µU reverse transcriptase per millilitre of undiluted cell supernatant or serum sample.

Results

Porcine kidneys and human vessel grafts survived up to 19 days after transplantation into cynomolgus monkeys. As described in Material and methods, six cynomolgus monkeys were transplanted with non-transgenic and CD59 transgenic kidneys. In order to analyse potential PERV in vivo transmission to human endothelium in a clinical-like whole-organ xenotransplantation setting, human vascular interposition grafts constructed from two vena saphena fragments, were placed distal to the porcine kidney graft (Figs. 1 and 2). To achieve delayed rejection of the porcine kidney and to guarantee survival of the human vascular graft, established immunosuppressive protocols, including CyP induction therapy, CyA, low levels of steroids and C1-Inh were applied. Porcine grafts, as well as human interposition grafts, were removed after evidence for steroid-resistant graft rejection had been obtained. Survival times were 2, 4, 6, 13, 16 and 19 days (Table 1); three of the animals died from septicaemia, with positive blood cultures indicating over-immunosuppression (Table 1).

Demonstration of an intact human endothelial cell layer in the interposition grafts

The transplantation of a human graft into a non-human primate recipient represents a concordant xenotransplantation. Since our immunosuppressive protocol was able to prevent rejection of the discordant porcine graft, we expected it to act in an equal manner with respect to the human interposition graft. Nevertheless, we checked the explanted human grafts for an intact human endothelium by immunohistochemical staining with a monoclonal antibody that is specific for human CD31 and does not cross-react with the cynomolgus or porcine counterpart. The endothelial cell layers of all explanted grafts that were analysed stained positive with the mAb specific for human CD31 (Table 1, Fig. 3). Controls without primary antibody and staining of cynomolgus vein sections were negative (Fig. 3A, B), whereas the human control vein (Fig. 3C) was positive.

Low proportions of porcine cells in endothelial cell cultures derived from human interposition grafts

Isolation of huECs from all explanted human interposition grafts was possible. The resulting huEC cultures were analysed by flow cytometry with antibodies specific for endothelial cells (anti-CD31) and fibroblasts (anti-CD90). We observed that 85% to 98% of the cells of all tested cultures stained positive for CD31 and contained

Table 1 Overview of the transplanted animals, grafts and results (*AVR* acute vascular rejection,*n.d.* not determined, *P* passage, *RT* reverse transcriptase/reverse transcription. Assessment of immunohistochemical staining: +> 80% of the endothelial cells clearly stained positive, ++ > 95% of the endothelial cells stained positive. FACS analysis: + a detectable proportion of cells stained positive)

Recipient animal	192	57B	75	679	813	673
Porcine graft	Non-transgenic	Non-transgenic	Non-transgenic	huCD59-transgenic	huCD59-transgenic	huCD59-transgenic
Survival time	6 days	13 days	16 days	19 days	2 days	4 days
Cause of death	Septicaemia[a]	Septicaemia[a]	Septicaemia[a]	AVR	Cardiovascular[c]	Technical[b]
Interposition graft/isolated cells						
Immunohistochemistry: anti human CD31	++	++	n.d.	+	+	++
FACS: Gal-α1,3-Gal specific IB4	+	> 4. P. Negative	Negative	Negative	Negative	Negative
PERV-specific PCR	+	> 5. P. Negative	Negative	Negative	Negative	Negative
Pig-specific PCR	+	> 5. P. Negative	Negative	Negative	Negative	Negative
RT-assay (culture supernatant)	Negative	Negative	Negative	Negative	Negative	Negative
Cynomolgus PBL						
PERV-specific PCR	Negative	Negative	Negative	Negative	Negative	Negative
Cynomolgus plasma/sera						
RT-assay	Negative	Negative	Negative	Negative	Negative	Negative
RT-PCR	Negative	Negative	Negative	Negative	Negative	Negative
Western blot analysis	Negative	Negative	Negative	Negative	Negative	Negative

[a]Fulminant bacterial septicaemia with positive blood cultures
[b]Thrombosis of the interposition graft
[c]Postoperative arrhythmia following extubation

between 2% and 8% contaminating fibroblasts (data not shown).

Moreover, the huEC cultures were screened for contaminating porcine cells by flow cytometry. The Gal-α1,3-Gal-specific lectin IB4 was used to identify porcine cells within the huEC cultures. Only two of the tested cultures showed very low contamination with porcine cells near the flow cytometry detection limit (Table 1). Figure 4 shows representative results of the endothelial cell (EC) cultures. These results were confirmed by PCR, supplying further evidence for low-level contamination with pig cells and/or pig DNA (Table 1).

No evidence for infection in the human grafts or in cultured human endothelial cells isolated from human interposition grafts

Tissue lysates of all explanted interposition grafts, as well as lysates of graft-derived huEC cultures, were analysed for PERV transmission by PERV pol-specific PCR. All samples that yielded ambiguous results were analysed by a second PERV-specific primer pair. As depicted in Table 1, tissue samples and cell culture lysates generated from human interposition grafts of animals 75, 679, 813 and 673 were negative in the PERV-specific PCRs. In accordance with the flow cytometry data, no pig-specific sequences could be detected in most samples.

By contrast, PERV pol and pig CyO II/centromere-specific PCRs were positive in the human interposition grafts explanted from recipients 192 and 57B. Whereas huECs derived from recipient 192 were positive in both PERV and pig-specific PCRs up to passage 10, cells isolated from the human graft of animal 57B were negative from passage 6 on. In order to exclude PERV infection, especially in animals 192 and 57B, quantitative real-time PCRs specific for PERV pol and pig SLA were performed. The resulting ratios PERV pol/SLA were compared with the corresponding ratios obtained from PBLs of the donor pigs and did not provide any evidence for PERV infection (data not shown). To support the PCR results, expression of PERV mRNA in the cultured huECs and release of reverse transcriptase activity into the cell culture supernatants was analysed; no evidence for productive PERV infection was obtained (data not shown).

No indication for productive PERV infection of the recipient animals

Productive retroviral infection is characterised not only by viral expression but also by release of virions. Usually virus-specific RT–PCR, or detection of reverse transcriptase in serum or plasma samples, is used to

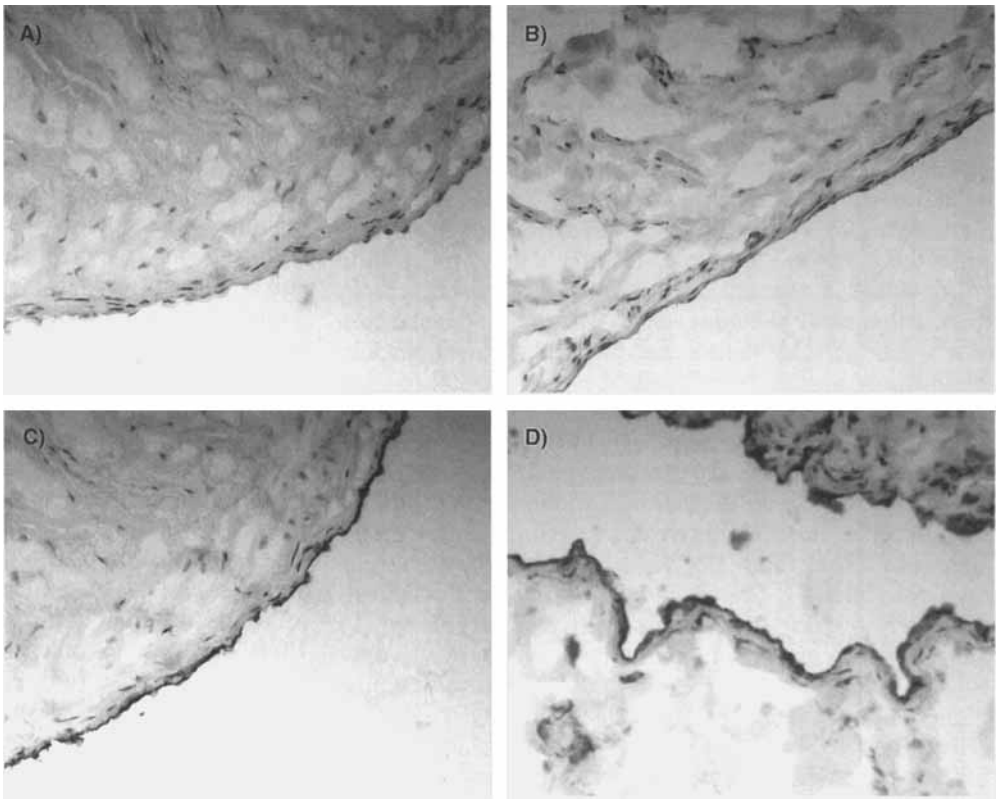

Fig. 3A–D Immunohistochemical demonstration of intact human endothelial layer in the explanted interposition grafts. **A** Human vein, negative control without primary antibody. **B** Vein of *Macaca fascicularis*, stained with antibody specific for huCD31. **C** Human vein, stained with antibody specific for huCD31. **D** Interposition graft (animal 192), stained with antibody specific for huCD31

prove productive infection of the analysed individual. To exclude productive infection of the recipient cynomolgus monkeys we screened plasma samples of all animals for PERV mRNA and sera for reverse transcriptase activity. No PERV mRNA or reverse transcriptase activity could be demonstrated (data not shown).

Production of specific anti-viral antibodies represents another indication for productive PERV infection. We tested serum samples of the recipient animals collected between day 1 and day 19 after transplantation for PERV-specific immune response. Using a highly sensitive PERV-specific Western blot analysis, determined by control PERV-specific animal sera, we could detect no specific anti-PERV antibodies in the tested sera (Fig. 5).

Discussion

It is now undisputed that pig cell lines and primary pig cells isolated from most pig breeds release humanotropic PERV in vitro [11]. Retrospective studies on patients and infection experiments in vivo revealed differing results [10, 31]. Neither the reported retrospective studies on patients nor in vivo infection experiments in SCID mice resemble a putative clinical whole-organ xenotransplantation setting. Experimental transplantation of pig organs into non-human primates may mirror clinical xenotransplantation much more closely. None of the recent studies analysing potential in vivo infection of non-human primates resulted in evidence of PERV infection [18, 19, 20, 21]. However, it is controversial whether a potentially lower susceptibility of non-human primate cells than that of human ones accounts for the absence of PERV infection in these studies.

In our model, porcine virions released by the porcine kidney would immediately achieve close contact with endothelial cells of the human interposition graft. This cell type, lining all human vessels, has been shown to be permissive to productive PERV infection [6].

Recipient animals survived for between 2 days and 19 days with intact, urine-producing xenografts. In this life-supporting model of kidney xenotransplantation, normal recipient creatinine levels indicate an intact graft blood flow (including the interposition graft). As the contact between porcine graft, recipient circulation and human endothelium lasted several weeks (at least in animals 57b, 75 and 679) these results should be highly comparable to future clinical application of xenotransplants for the bridging of acute organ failure. It could be argued that 2 weeks of in vivo exposition followed by at least 4 weeks of ex vivo cultivation of the isolated endothelial cells should be a sufficient time frame for productive PERV infection to develop or, at least, for PERV integration to occur. Nevertheless, longer exposition times and follow-up periods would be desirable.

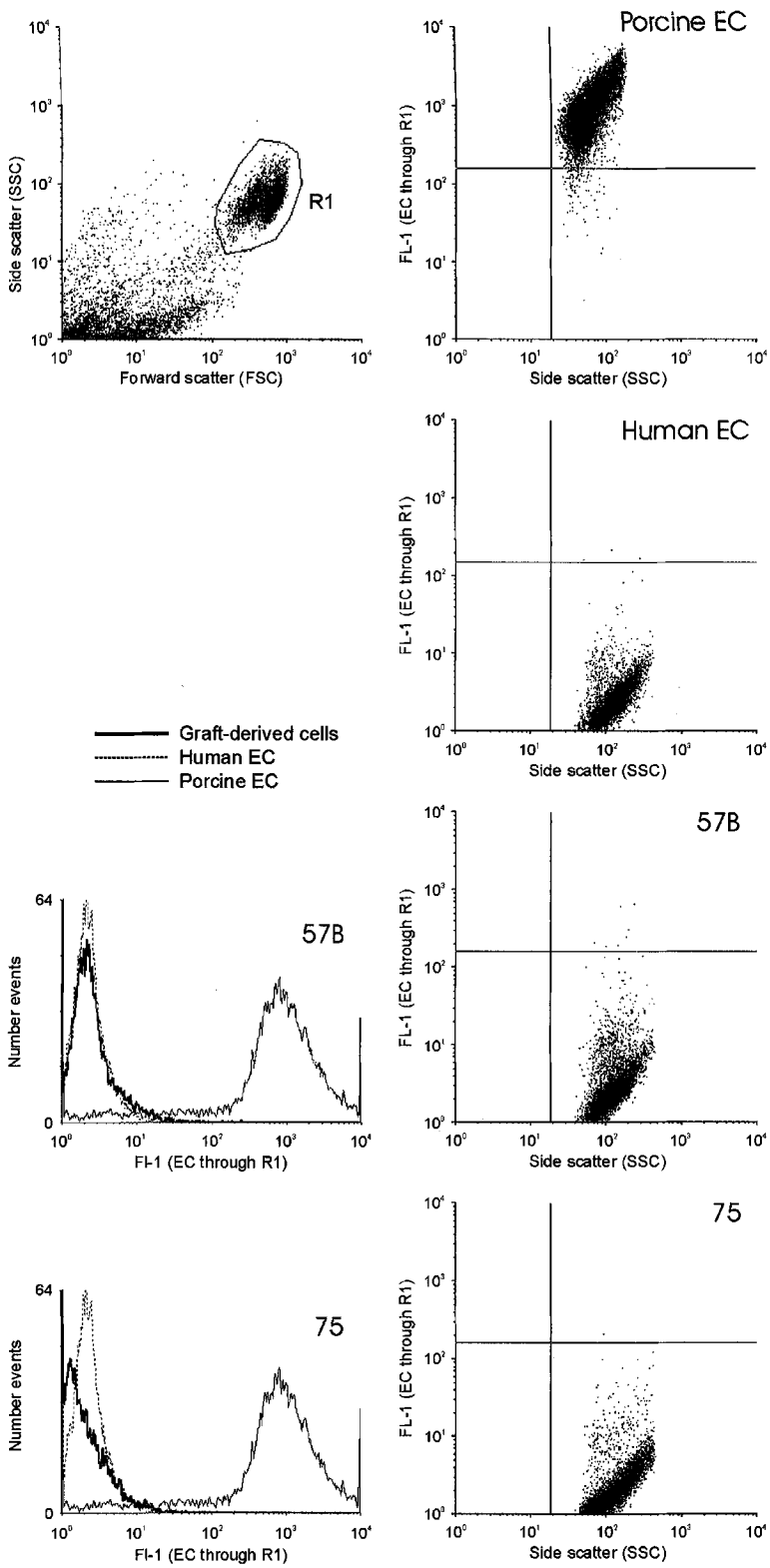

Fig. 4 Low proportions of contaminating porcine cells in graft-derived human endothelial cell cultures as demonstrated by FACS analysis. Interposition graft derived huEC cultures (animal 57B and 75) in the second passage have been stained by Gal-α1,3-Gal-specific IB4 and analysed by flow cytometry. Cells isolated from the interposition graft of animal 57B contained a very low proportion of contaminating porcine cells, whereas no cells derived from the interposition graft of animal 75 stained positive with IB4. Human and porcine endothelial cells served as controls

Similar to those in possible future clinical xenotransplantation settings, cellular and humoral mechanisms of the recipient's immune system against foreign viruses are impaired, in our model, by the combination therapy of CyP induction, CyA, low-dose steroids and mycophenolic acid. The use of huCD59 transgenic

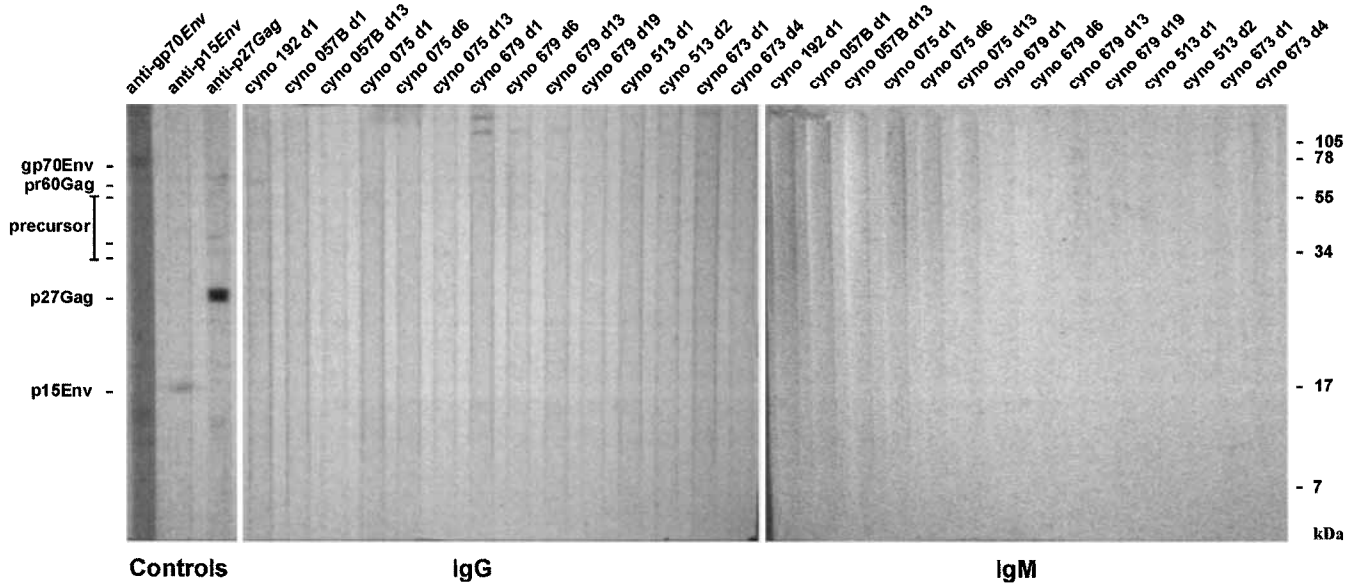

Fig. 5 No evidence for PERV-specific immune response in the recipient animals as demonstrated by Western blot analysis. Cynomolgus sera (1:100) were screened for PERV-specific IgM and IgG immune responses. Purified viral protein from infected HEK293 cells (PERV/5°) was separated by PAGE and blotted. Detection was performed with a peroxidase-coupled anti-human IgM antibody at 1:1,000 or, alternatively, by a 1:5,000 dilution of peroxidase-coupled protein G. Anti-gp70Env, anti-pr60Gag and anti-p27Gag antibodies, conjugated with HRP (1:1,000) and detected with anti-goat IgG, were used as positive controls

porcine grafts and the administration of C1-esterase inhibitor further affects, in particular, the complement-mediated destruction of potentially released PERV particles in the animals' sera.

In the experiments described three out of six animals died from infective complications, and only one animal died from (acute vascular) rejection. This clearly indicates that the multi-drug regimen administered to the animals induced profound and heavy immunosuppression, which, in this model, was the desired aim of the experiments. Despite up to 19 days of lasting contact with functional porcine kidneys, and despite clinical over-immunosuppression of the recipient animals, no evidence for PERV infection of the recipients was observed. In fact, we were unable to demonstrate PERV particles in the recipients' serum samples by detection of reverse transcriptase activity. In agreement with a former study [18], no PERV mRNA could be detected, although the approach we used is sensitive enough to detect PERV RNA in pig serum. These results may still be due to insufficient sensitivity of the assay or to relatively quick destruction of virions by the recipients' immune systems.

To investigate thoroughly potential PERV transmission to endothelial cells of the human vessel grafts, it was necessary to isolate huECs from the interposition grafts. The integrity of the vascular endothelial cell layer is maintained by proliferation of local endothelial cells as well as by circulating endothelial cells and endothelial cell progenitors (EPCs) [32]. It has been reported that endothelial cells of a vascularised graft can be replaced by cells of the recipient, derived from circulating endothelial cells or EPCs [33]. To exclude the possibility that the human endothelium had been replaced, we analysed the explanted human grafts by appropriate immunohistological methods. Application of an anti-human CD31 antibody not cross-reacting with pig or cynomolgus CD31 clearly demonstrated an intact huEC layer in all explanted grafts (Fig. 3). Moreover, endothelial cell cultures derived from the human interposition grafts were checked flow cytometry for contaminating pig cells using the Gal-α1,3-Gal-specific lectin IB4 (Fig. 4). Contaminating porcine cells were detected only in cultures derived from interponates of animals 192 and 57B, probably due to inaccurate resection from the porcine kidney vein during explantation of the human graft (Table 1). Although the cell cultures contained between 2% and 8% of contaminating fibroblasts (data not shown), we were able to demonstrate that the analysed cell cultures comprised a majority of endothelial cells permissive for productive PERV infection [6].

All interposition grafts and cell cultures were analysed by two different highly sensitive PERV pol-specific PCRs. The majority of samples were clearly negative in both assays as well as in pig CyOII and pig centromere-specific PCRs. All samples, which yielded positive results in the PERV-specific PCRs, were also positive in the pig-specific PCRs. To exclude infection in those samples, we performed real-time PCRs specific for PERV pol and pig SLA (data not shown). Comparison of the resulting PERV pol/pig SLA ratios of the respective sample with the ratios obtained for the donor pigs clearly argued

against an infection but for low contamination with porcine cells or porcine DNA. Our PCR results were supported by the fact that no PERV RNA and no reverse transcriptase activity could be detected in the cell cultures (Table 1).

Evidence for the presence of intact PERV particles in the porcine blood stream under in vivo conditions has been presented by two different groups [4, 9]. As this was not the aim of our study, we did not investigate whether, in our model, PERV particles were released from the xenograft after transplantation. In view of the fact that during our experiments xenograft endothelial cells, able to release infectious virus in vitro [5], did undergo constant and numerous immunological attacks (e.g. by preformed/induced recipient antibody deposition), a profound endothelial cell activation resulting in release of PERV virions into the recipient circulation has to be assumed.

Our data clearly indicate that no PERV infection occurs in a pre-clinical xenotransplantation setting closely resembling clinical whole-organ xenotransplantation. From a scientific point of view, the underlying reasons for the absence of PERV infection have to be discussed and determined in future studies.

As indicated above, absence of PERV release from the xenograft is one potential explanation for the failure to detect PERV infection of the human graft. Another reason may be that the remaining immunological competence of the recipient still guaranteed immediate elimination of PERVs, in spite of profound pharmacological immunosuppression and complement inhibition. We cannot decide whether absence of PERV release or immediate PERV destruction accounts for the absence of PERV infection in our model; however, both mechanisms would be acceptable from a clinical point of view.

In conclusion, this is the first infection model suitable to analyse the risk of PERV transmission to human tissue during clinical-like whole-organ pig-to-primate xenotransplantation. A variety of different assays revealed that PERV transmission to the recipients or to the co-transplanted human vascular tissue did not occur, although huCD59 transgenic organs had been transplanted and although the animals had undergone continuous heavy immunosuppression and complement inhibition.

Besides its value in PERV research, our model may be the basis for future infection studies focussing on other pig-derived viruses and pathogens that can be transmitted after xenotransplantation of pig organs.

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