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Porcine endogenous retrovirus (PERV) was not transmitted from transplanted porcine endothelial cells to baboons in vivo

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Abstract The discussion about the clinical risk of zoonoses in xenotransplantation has recently culminated in the demand for a moratorium on clinical organ transplantation using pig donors. The basis for this discussion was a recent report showing a possible trans-species transmission of pig endogenous retrovirus (PERV) by in vitro transfer to human cell lines. At present, it remains unclear if this could also happen in vivo or in the setting of xenotransplantation. Potential in vivo transfer of PERV after xenotransplantation was investigated in an experimental pig-to-baboon cell transplantation model. Baboons were immunosuppressed with high-dose cyclophosphamide (total 45–150 mg/kg) and transplanted with primary porcine aortic endothelial cells (PAEC). Tissue samples (skin, lymph nodes, lung) and peripheral blood leukocytes of 15 baboons, taken about 12–24 months after transplantation of PAEC, were then analyzed by PCR and showed no PERV infection. PERV expression in PAEC was also analyzed: PERV mRNA and reverse transcriptase in the culture supernatant could be detected. In spite of the release of retroviral particles from cultured PAEC, transplantation of these cells into baboon recipients did not result in virus transmission, not even under heavy immunosuppression.

Key words Xenotransplantation, retrovirus · Retrovirus, xenotransplantation · PERV · Pig endothelial cells · baboon

Introduction

The shortage of human organs for transplantation has led to considerable efforts to transplant animal-derived organs into human beings. Success in overcoming hyperacute rejection in transgenic animals has recently raised the prospect of clinical introduction of discordant xenotransplantation using pig donors. The potential risk of zoonosis was thought to be low in discordant species like the pig. Last year, however, Patience et al. were able to show that porcine kidney cell lines PK15 and MPK release particles of pig endogenous retrovirus (PERV), which are able to infect human cell lines in vitro [7].

It is as yet unknown whether the findings of Patience et al. represent in vitro artifacts or whether infection can occur in the normal in vivo situation. The question also remains whether the transmission of PERV to normal, healthy humans is prevented by natural α-Gal binding antibodies and complement, as has been shown for oth-

er retroviruses [8, 9, 11]. If infection is possible, one needs to determine whether the virus is transmitted by blood contact and/or by food, and why considerable segments of the human population are not PERV-positive. Also unknown is whether infection can occur in the setting of a xenotransplantation. One difference between such a setting and the normal in vivo situation may be the potentially lower titer of natural antibodies, including anti α-Gal antibodies, in the heavily immunosuppressed recipient. This could result in a diminished virolysis of PERV particles. Genetic modifications, such as the elimination of porcine Gal epitopes or the introduction of complement regulators, proposed or performed to overcome hyperacute rejection, could also influence the elimination of virions. Under these conditions, infection of human cells with PERV might be promoted [13].

Scientists, physicians, and ethicists are currently discussing whether first clinical trials should be postponed due to the risks of xenozoonosis [1, 2, 5, 12, 13]. Bach and co-authors called for a moratorium in xenotransplantation [3, 4]; they favor continuing basic research, especially in the field of xenozoonosis. Before first clinical trials are started, it seems that a public debate about the risks and ethics of xenotransplantation should take place.

In this study, we addressed the question of potential in vivo transfer of PERV after xenotransplantation in an experimental pig-to-baboon cell transplantation model. We also investigated whether primary porcine endothelial cells express PERV mRNA or release retrovirus particles.

Methods

Porcine endothelial cells

Porcine aortic endothelial cells (PAEC) were isolated by incubating the inner layer of porcine aortas with 0.2% collagenase type II (Boehringer Mannheim, Germany) for 20 min at 37 °C. Detached endothelial cells were washed twice in PBS and subsequently cultured in M199 (Biochrom, Berlin, Germany) plus 20% FCS (Life Technologies, Eggenstein, Germany) and ECGF (Boehringer Mannheim, Germany). The cells were harvested by incubation with 0.05 M trypsin/0.02 M EDTA and tested for viability (trypan blue). Cells were prepared for transplantation in a volume of 10 ml PBS.

Transplantation of the porcine endothelial cells into immunosuppressed baboon recipients (*Papio hamadryas*)

Sixteen baboons (4–10 years old, 9–24 kg) were transplanted one or (in three cases) two times with 1×10^7 PAEC by slow i.v. injection. One of the baboons had a pigheart transplanted heterotopically but experienced hyperacute rejection after 40 min. These animals, except two control monkeys, were immunosuppressed with high-dose cyclophosphamide (15–45 mg / kg weight per day i.v.) on days 0–4 after transplantation. Except for one baboon that died of sepsis, all baboons survived and are healthy. Blood and tissue samples (leukocytes, skin, lymph nodes, and liver) of the baboons were collected after an interval of 12–24 months after primary PAEC transplantation.

Flow cytometric analyses of the baboon sera

Immunosuppression and antiporcine immune reaction were monitored by measuring the porcine-specific IgM and IgG titers on days –1, 0, 1, 2, 3, 4, 5, 10, 20, and 30. The baboon sera were analyzed by flow cytometry for their binding to porcine mononuclear cells.

PCR detection of porcine endogenous retrovirus (PERV) in cells and tissues

For PCR detection, 2×10^6 cells or 5 tissue sections (each 10 µm thick) were lysed in 200 µl of 200 µg / ml proteinase K in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM TRIS-HCl pH 8.4) for 3 h at 56 °C, followed by a 10-min inactivation of the enzyme at 95 °C. A volume of 3.5 µl of this crude extract served as a template for the PCRs, which were performed essentially as described by Patience et al. [7] with PERV protease-specific primers. Control PCRs were carried out according to Heneine and Switzer [6]. Determination of the PCR sensitivity was performed as follows. Different quantities of lysed PK15 cells were mixed with 1×10^6 lysed baboon leukocytes. These mixed crude extracts served as a template for PCRs, as described above.

Detection of PERV-specific mRNA by RT-PCR

Total RNA of PAECs was prepared using Tri Reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to the manufacturer's instructions. Contaminating DNA was digested by DNase treatment [10 µg total RNA, 10 U RNase-free DNase I (Stratagene, Heidelberg, Germany), 40 U/µl RNase inhibitor (Boehringer Mannheim, Germany), 1 M $MgCl_2$, and 0.1 M DTT] for 15 min at 37 °C, followed by a phenol/chloroform extraction. After sodium acetate precipitation, 2 µg of the RNA was used for random-primed cDNA synthesis with AMV-RT (Boehringer Mannheim, Germany), as described by the manufacturer. The following PERV protease-specific PCR was performed according to Patience et al. [7].

RT-PCR-based assay for reverse transferase activity in cell culture supernatant

Measurement of reverse transcriptase in the supernatant was performed as described by Patience et al. and Silver et al. [7, 10] with some modifications: 3 ml of 0.45-µm filtered cell culture supernatant was pelleted and resuspended in 20 µl 1% NP40. Five microliters of this solution was then tested in the RT-PCR-based assay. After reverse transcription (sequence of the RT primer according to Silver et al. [10] and subsequent denaturation at 95 °C for 5 min, the BMV-RNA was digested by 100 µg/µl RNase H (Boehringer Mannheim, Germany) for 15 min at 37 °C. The following PCR was performed as described by Silver et al. [10]. Unlike this protocol, reverse transcription and the subsequent PCR reaction occurred in different tubes. Subsequently, 18 µl of the PCR product was loaded on a 2% agarose gel. After separation, the DNA

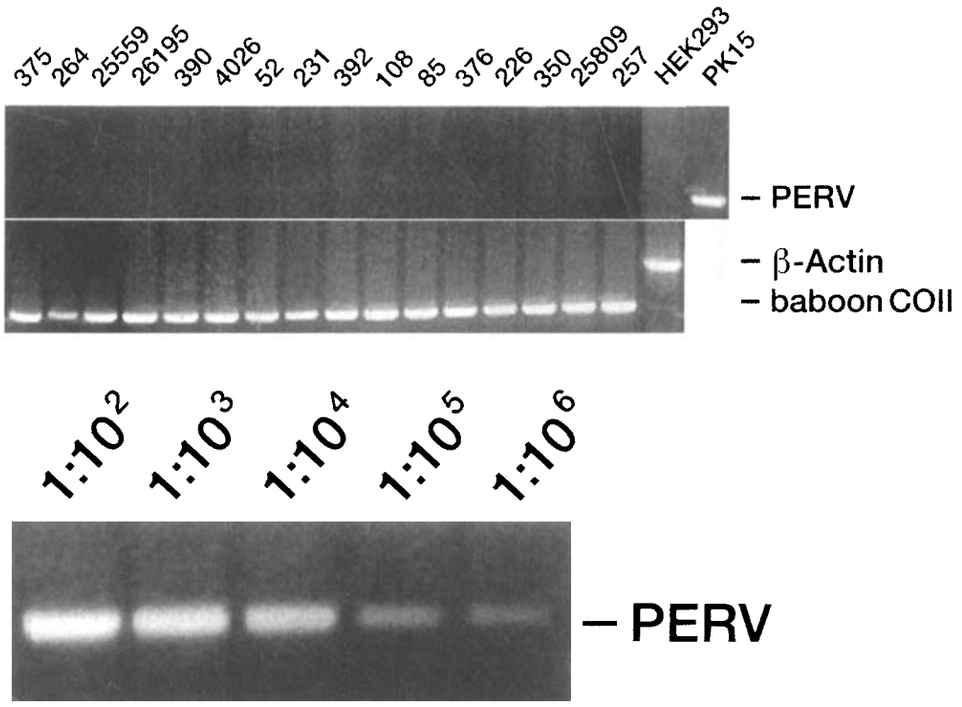

Fig. 1 a Analysis of leukocytes of the transplanted baboons by PERV protease-specific PCR (*upper lane*). Human HEK 293 cells and porcine PK15 cells were used as controls. Internal positive controls for each sample were performed by PCR specific for baboon cytochrome oxidase II or β-Actin in the case of human HEK293 cells (*lower lane*). **b** Sensitivity of the PERV protease-specific PCR determined by endpoint dilution of porcine PK15 cells in baboon leukocytes

was blotted in 20 × SSC on a nylon membrane (Hybond N, Amersham, Braunschweig, Germany), denatured on the membrane with 0.5 M NaOH, 1.5 M NaCl, neutralized in 3 M NaCl, 1 M TRIS-HCl pH 7.0 for 5 min each, and crosslinked by UV irradiation according to standard protocols.

An internal BMV-specific oligonucleotide (5'CAAGAGTG TCTAGGCGCCTTTGAGAGT-3') was labeled using the DIG-oligonucleotide tailing kit (Boehringer Mannheim, Germany). The blot was hybridized in 5 × SSC, 0.1% N-laurylsarcosine, 0.02% SDS, 1% blocking reagent (Boehringer Mannheim, Germany), 0.1 mg/ml Poly(A) (Boehringer Mannheim, Germany) at 50 °C ON with the DIG-tailed oligonucleotide, washed twice for 5 min at room temperature and twice for 15 min at 65 °C in 2 × SSC. Chemoluminescence detection was performed using the DIG luminescence detection kit (Boehringer Mannheim, Germany), according to the manufacturer's instructions.

Reagents

If not otherwise noted, all chemicals were purchased from Sigma Chemicals (Deisenhofen, Germany).

Results

Clinical course

Immunosuppressed baboons received high-dose cyclophosphamide (30–45 mg/kg per day on days 0–4) and showed a delayed onset of antiporcine IgM/IgG response starting on day 10 after PAEC transplantation (data not shown). After recovery of leukocyte and thrombocyte counts (30 days), the animals were kept under long-term medical observation and showed no signs of disease.

PERV-specific DNA sequences

Transplanted baboons were analyzed for the transmission of PERV using a PERV protease-specific PCR. Leukocytes and tissue samples (skin, lymph nodes, and liver) of 16 baboons were taken 1–2 years after transplantation of the porcine cells. The chimeric persistence of PAEC in tissues was excluded by a β-globin-specific PCR (data not shown).

As shown in Fig. 1 a, the tested leukocytes of all monkeys, including animals with low natural antibody titer before PAEC transplantation, were PERV-negative as determined by PERV protease-specific PCR. Likewise, all tested tissues of the baboons were PERV-negative (data not shown). Internal control PCRs specific for baboon cytochrome oxidase II were, in all cases, positive. The sensitivity of the assay was tested while porcine PK15 cells were mixed with baboon leukocytes in different dilutions. PERV sequences of one PK15 cell could be detected in 10^5–10^6 baboon leukocytes (Fig. 1 b).

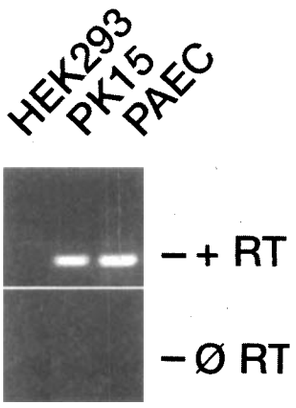

Fig. 2 Analysis of PERV expression by PK15 cells and PAEC using PERV protease-specific RT-PCR (*upper lane*). HEK293 cells were used as negative control. Internal controls without reverse transcriptase confirmed that genomic DNA was not amplified but that cDNA was (*lower lane*)

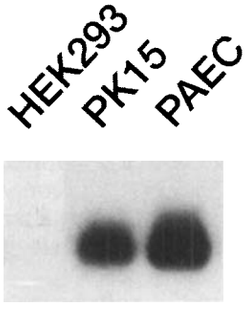

Fig. 3 Results of an RT-PCR-based assay for detecting reverse transcriptase in the supernatant of cultured porcine cells. Human HEK293 cells served as negative control

In order to examine whether there is at least in vitro transmission of PERV to baboon cells, we cocultured lethal x-irradiated PK15 cells or PAEC, respectively, with a baboon cell line. Using PERV protease-specific PCR, after the fifth passage PERV sequences could still be detected, whereas no more porcine β-globin DNA could be found (preliminary results, data not shown).

PERV expression by PAEC

In order to test whether the transplanted primary PAEC would express PERV mRNA, we prepared total RNA and performed RT-PCRs. Cultured PK15 cells and primary PAEC pooled from different pig donors were found to be positive for PERV protease mRNA, as shown in Fig. 2. Internal negative controls without reverse transcriptase proved that viral RNA, and not genomic DNA, was detected.

We also investigated whether there would be not only PERV mRNA expression in PAECs but also release of retroviral particles. Using an RT-PCR-based assay for detecting reverse transcriptase (RT) in the culture supernatant we were able to show RT activity in the supernatant of the porcine kidney cell line PK15 and also in the supernatant of primary PAEC (Fig. 3). In contrast, the human HEK 293 cell line was found to be negative for RT.

Discussion

The current discussion about the potential risks of zoonosis reflects the great importance of this issue for the whole field of xenotransplantation. In particular, the transmission of PERV to humans gained tremendous attention after the report of in vitro transmission to human cells [7]. Because of the variety of potentially dire consequences after clinical transplantation into immunosuppressed recipients, including the risks of patient disease and epidemic transmission of humanized viruses, subhuman models are needed to evaluate this question and to calculate the clinical risk of xenotransplantation. In this study, we examined the possibility of PERV transmission by xenotransplantation in a pig-to-baboon cell transplantation model using endothelial cells.

Endothelial cells are the main interface of a xenogenic organ to host immune cells and may be a main source of retroviruses infecting host leukocytes upon adhesive contact. It was not known whether the release of PERV by porcine cells is only a cell line artifact or whether it also occurs in the case of primary porcine cells. Our in vitro studies clearly show that primary PAEC also express PERV protease and are able to release retrovirus particles in the supernatant, at least under cell culture conditions. Further experiments have to be done in the future to clarify the in vivo expression and release of PERV.

PCR analysis 12–24 months after in vivo transplantation of PAEC showed an absence of PERV sequences in all baboon tissues tested (PBL, lymph nodes, liver, skin), as well as an absence of porcine-specific sequences excluding chimeric persistence of PAEC.

Given this experimental setup, the question arises as to whether the time PAEC persisted was long enough to permit PERV transmission to baboon cells. It can be assumed that a primary cytolysis of PAEC in the circulation can be exerted even in the presence of low or moderate titers of natural antibodies. One could speculate that the initiation of immunosuppression after transplantation was too late to decrease the titer of natural antibodies and to prevent elimination of PAEC or PERV particles. However, analysis of the baboon sera showed that the high-dose cyclophosphamide immunosuppression did not reduce the titer of natural anti-porcine antibodies, not even at later time points.

On the other hand, a delayed onset of IgM and IgG antiporcine antibody response was observed in baboons receiving high-dose cyclophosphamide immunosuppression starting 5–10 days after transplantation. This may point to a persistence of some of the transplanted PAEC or released retroviral particles in the tissue or cir-

culation up to at least several days after transplantation. No studies of chimeric persistence of PAEC were performed in the initial phase after transplantation. Additional studies are needed to clarify the primary elimination of transplanted PAEC and PERV particles by natural antibodies and complement. Further in vitro infection experiments with different cell types from baboons, cynomolgus, and other old world monkeys should be performed to elucidate the suitability of these species for subhuman in vivo infection models.

The potential modification of in vivo PERV transmission by the preabsorption of xenophile antibodies or depletion of complement should be examined in future cell transplantation experiments.

In conclusion, this is the first report showing that not only porcine cell lines but also primary porcine cells are able to express PERV mRNA and also to release retroviral particles. We were also able to show that in our experimental pig-to-baboon model transplantation of PERV-releasing endothelial cells to highly immunosuppressed baboons did not result in transmission of PERV.

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