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Virus safety of islet cell transplantation from transgenic pigs to marmosets

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ABSTRACT

Transplantation of pig islet cells for the treatment of diabetes may be a more effective approach compared with the application of insulin. However, before introduction into the clinic, efficacy and safety of this treatment have to be shown. Non-human primate models may be used for this, despite the fact that they are characterised by several limitations. Here we investigate the prevalence of porcine endogenous retroviruses (PERVs), which are present in the genome of all pigs and which may infect human cells, as well as of porcine herpes viruses in donor pigs and their potential transmission to non-human primate recipients. Despite the fact that all three subtypes of PERV were present in all and porcine cytomegalovirus (PCMV) was found in some of the pigs, neither PERVs nor PCMV were found in the recipient animals under the experimental conditions applied. Porcine lymphotropic herpes viruses (PLHV) were not found in the donor pigs, hepatitis E virus (HEV) was not found in the recipients.

1. Introduction

As at 2013, 387 million people have diabetes worldwide (International Diabetes Federation, 2014). This is equal to 8.3% of the adult population. For comparison, there are 35 million individuals infected with HIV-1 worldwide. The expected increase in the number of diabetes patients by 2035 is 205 million, summing up in 592 million. Due to the non-optimal application of insulin, late effects such as blindness, amputation and cardiovascular diseases are the reasons for most of the costs spent for diabetes treatment. Diabetes mellitus type 1 (previously called insulin-dependent diabetes) accounts for 5% of cases of diabetes (American Diabetes Association. Type 1 Diabetes). The prevalence of insulin-dependent diabetes in the United States is estimated to be 300,000–500,000 individuals of all ages (LaPorte et al., 2013). World-wide more than a 50-fold geographic variation in the annual incidence was observed ranging from 0.7 per 100,000 in Shanghai and 35.3 per 100,000 in Finland. There is evidence that insulin-dependent diabetes is increasing globally (LaPorte et al., 2013). For the treatment of type 1 diabetes, xenotransplantation of pig islet cells with biologically regulated insulin release may be a better approach.

Regarding safety, xenotransplantation may be associated with transmission of porcine zoonotic microorganism (Fishman et al., 2012; Mueller et al., 2011). Most porcine microorganisms may be eliminated from the donor animals by specified pathogen-free (spf) maintenance and breeding, but porcine endogenous retroviruses (PERVs) are thought to pose the highest risk, since they are integrated in the genome of all pigs (for review see Denner and Tönjens, 2012). PERV-A and PERV-B are present in all pigs and they are able to infect human (mainly tumour) cells *in vitro*. PERV-C is not present in all pigs and it infects only pig cells. Since PERV-C may recombine with PERV-A resulting in high titre PERV-A/C infecting human cells, under certain conditions even primary cells (Costa et al., 2014;

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Denner, 2014), it was recommended not to use pigs with PERV-C in their genome for xenotransplantation. However, it is unclear whether the transmitting phenotype is a stable or an intermittent trait (Martin et al., 2006). Until now no PERV transmission was observed in all preclinical and clinical xenotransplantations (for review see Denner and Tönjens, 2012). Meanwhile it became clear that other viruses, which do not integrate into the cellular genome, such as hepatitis E virus (HEV) (Busby et al., 2013; Denner, 2015) and porcine herpes viruses (Mueller and Fishman, 2004) are difficult to detect and may be difficult to eliminate. Presently, there is no experience with the elimination of these viruses from infected herds. Importantly and in addition to safety issues, transmission of PCMV together with the transplant from pigs to non-human primates significantly reduced the survival time of a pig xenotransplant in the recipients (Sekijima et al., 2014; Yamada et al., 2014).

Here we investigate the prevalence of PERVs and porcine herpes viruses in German landrace pigs, either in wild-type animals or in transgenic pigs expressing LEA29Y, a high-affinity variant of the T-cell costimulation inhibitor CTLA-4Ig under the control of the porcine insulin gene promoter (Klymiuk et al., 2012). After transplantation of islet cells from these animals, transmission of the porcine viruses to the xenotransplant recipient, common marmoset (*Callithrix jacchus*), was studied.

2. Materials and methods

2.1. Donor animals, transplantation, recipients

Fourteen piglets either non-transgenic or expressing *INS*LEA29Y (German landrace hybrid pigs bred at the Chair for Molecular Animal Breeding and Biotechnology, LMU, Munich) (age 2–5 days) were analysed for the prevalence of different PERVs in the genome and for the presence of porcine herpes viruses. Neonatal islet like cell clusters (ICCs) were isolated as described previously (Klymiuk et al., 2012) and transplanted into normoglycaemic marmosets (*C. jacchus*) (German Primate Center, Göttingen) (body weight 411–451 g, #14016, 7 years old, male; #14942, 5 years old, male; #14862, 5 years old, female; #15349, 4 years old, female) by intramuscular injection into the *Musculus vastus medialis* (25,000–30,000 ICCs per animal). No systemic immunosuppressive therapy was administered. After 6 months, the recipients of the islet cell transplantation were investigated for transmission of the viruses found in the pigs and in addition for hepatitis E virus (HEV).

2.2. DNA and RNA isolation

DNA was isolated from the spleen of the donor pigs using the Wizard® Genomic DNA Purification Kit (Promega, Mannheim, Germany) according to the manufacturer's instruction. DNA from different organs including *Musculus quadriceps*, lung, liver, kidney, pancreas, small intestine and heart of the four recipient marmosets was isolated using the DNeasy® blood and tissue kit (Qiagen, Hilden, Germany). RNA was isolated from these organs using the RNeasy® Minikit (Qiagen) according to the manufacturer's instructions. The amount of DNA and RNA was quantified using a NanoDrop spectrophotometer.

2.3. PCR for the detection of porcine herpes viruses

The PCR methods for the detection of PCMV (Goltz et al., 2000), PLHV-1, PLHV-2 (Ehlers et al., unpublished data), and PLHV-3 (Chmielewicz et al., 2003) were performed as described, for primers see Table 1. Amplifications were performed after complete activation of the DNA Polymerase AmpliTaq Gold® for 12 min at 95 °C and then cycled 45 times through 30 s denaturation at 95 °C, 30 s at 55 °C (Primers 199-S/199-AS, PCMV), 56 °C (Primers 747-S/747-AS, PLHV-1, -2) or 57 °C (Primers 905-S/905-AS, PLHV-3) and extension between 1 and 2 min at 72 °C depending on amplicon size, followed by an extension step for 15 min at 72 °C. A 1.2% agarose and the GeneRuler™ 100 bp DNA ladder (Thermo Scientific, Schwerte, Germany) were used for gel electrophoresis.

2.4. PCR for the amplification of the marmoset PERV receptor

Amplifications were performed marmoset DNA, the Kapa2G robust polymerase (Peqlab Biotech. GmbH, Erlangen, Germany) and primers (Table 1) for 10 min at 94 °C and then cycled 35 times through 30 s denaturation at 94 °C, 30 s at 57 °C and for 40 s at 72 °C followed by an extension step for 5 min at 72 °C. A 1.2% agarose gel and the GeneRuler™ 100 bp DNA ladder (Thermo Scientific, Schwerte, Germany) were used for gel electrophoresis.

2.5. Nucleotide sequence analysis

Sequencing was performed bidirectionally using Sanger's dideoxy method with the Big Dye® Terminator sequencing kit (Invitrogen, Life Technologies, USA), using 10 ng of amplicon, 5 pmol of corresponding primers, 1 μl of 5× buffer and 2 μl of BigDye. The BLAST® program (NCBI) was used for database research and sequence alignments were obtained with the ClustalW module of Lasergene Version 10 (DNASTAR, Inc. Madison, USA).

2.6. PCR and real-time PCR for the detection of PERV and HEV

The methods for the amplification of PERV*gag*, PERV-C*env* and a new variant of PERV-C (PERV-Cnv*env*) were used as described (Kaulitz et al., 2011a,b, 2013) (Table 1). Duplex real-time PCRs were performed using porcine cyclophilin (cyp) as reference gene and the primers cyp fw and cyp rev and a cyp probe (Table 1). The thermal cycling conditions for PERV*gag* used were 10 min at 95 °C, followed by 45 cycles of 45 s denaturation at 95 °C, 1 min annealing at 55 °C and 30 s extension at 72 °C and for PERV-C*env* and PERV-Cnv*env* were 7 min at 95 °C followed by 45 cycles of 95 °C for 20 s and 58 °C for 30 s in a Stratagene MX3005P machine (Agilent, Waldbronn, Germany). For the estimation of the copy number of PERV-C in the donor pigs the amplicon of a PERV-C real-time PCR was cloned into the pBlueskript KSII vector (Stratagene California, La Jolla, USA). Positive clones were selected by colony PCR using the primers of the T7-promotor and M13 reverse (Table 1). For the detection of HEV a sensitive reverse transcriptase real-time PCR (Jothikumar et al., 2006) was used and amplification was performed using SensiFast™ probe no ROX one tube kit according to the manufacturer's instructions (Bioline, Luckenwalde, Germany) with the following thermal cycling conditions: reverse transcription for 35 min at 50 °C, denaturation at 95 °C for 15 min following 45 cycles with denaturation for 10 sec at 95 °C, annealing for 20 sec at 55 °C, and extension for 15 s at 72 °C. DNA from HEV-infected and PERV-positive pigs was used as positive controls.

2.7. Screening for antibodies: SDS–PAGE and Western blot analysis

Screening for PERV-specific antibodies was performed by Western blot analyses using virus lysate or viral recombinant proteins. The proteins used in the Western blot analysis were characterised by SDS–PAGE and stained with Coomassie Blue G-250. For the determination of the molecular weight the PageRuler™ prestained protein ladder (Thermo Scientific, Schwerte, Germany) was used. 500 ng rp15E (Fiebig et al., 2003), rp27Gag (Irgang et al., 2003) and rgp70/p52 (Kaulitz et al., 2011a,b) as well as 350 ng of a recombinant genotype 3 (GT3) ORF 2-HEV antigen (Dremsek et al.,