Susceptibility of Human Liver Cells to Porcine Endogenous Retrovirus

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Abstract
Objectives: The risk of porcine endogenous retrovirus infection is a major barrier for pig-to-human xenotransplant. Porcine endogenous retrovirus, present in porcine cells, can infect many human and nonhuman primate cells in vitro, but there is no evidence available about in vitro infection of human liver cells. We investigated the susceptibility of different human liver cells to porcine endogenous retrovirus.

Materials and Methods: The supernatant from a porcine kidney cell line was added to human liver cells, including a normal hepatocyte cell line (HL-7702 cells), primary hepatocytes (Phh cells), and a liver stellate cell line (Lx-2 cells), and to human embryonic kidney cells as a reference control. Expression of the porcine endogenous retrovirus antigen p15E in the human cells was evaluated with polymerase chain reaction, reverse transcription-polymerase chain reaction, and Western blot.

Results: The porcine endogenous retrovirus antigen p15E was not expressed in any human liver cells (HL-7702, Phh, or Lx-2 cells) that had been exposed to supernatants from porcine kidney cell lines. Porcine endogenous retrovirus-specific fragments were amplified in human kidney cells.

Conclusions: Human liver cells tested were not susceptible to infection by porcine endogenous retrovirus. Therefore, not all human cells are susceptible to porcine endogenous retrovirus.

Key words: Xenotransplant, Infection, Hepatocyte, Graft

Introduction

Xenotransplant brings hopes to patients with end-stage organ failure. Swines are the first choice for xenotransplant. However, the porcine endogenous retrovirus (PERV) present in porcine cells is an integral part of the porcine genome. This virus may copy or recombine as a new virus in transplant recipients, especially in those who receive potent immunosuppressant drugs. Although this recombination is rare, microchimeric PERV may exist in transplant recipients for a long time. It is unknown whether human cells can be infected by PERV in vivo or in vitro.

Transcription activity of PERV is present after porcine pancreatic islets are transplanted into mice that have severe combined immunodeficiency. These results presume that patients may be infected with PERV only when they have received porcine pancreatic islet cell transplant or immunosuppressant drugs. Additionally, baboon, monkey, and other nonhuman primates have been used as a model for infection by PERV. These results may not provide full characterization of human susceptibility to PERV because of insufficient exposure to PERV or inadequate immunosuppression of experimental animals.

Analysis of human cells from different tissues in vitro may provide information about the susceptibility of human cells to PERV infection and the potential that PERV may infect the human body in vivo.
studies have shown that PERV could infect different human cell lines, including T cells, bone marrow cells, natural killer cells, and kidney cells. This suggests that PERV may be transmitted to human cells. However, these studies were limited to cell lines and the circulatory system, and studies on cells from human solid organs, such as the liver, are not available.

In this study, we selected human embryonic kidney (HEK293) cells for a reference measure of the susceptibility of human cells to PERV. We chose different types of human liver cells, including a normal hepatocyte cell line (HL-7702 cells), primary hepatocytes (Phh cells), and a liver stellate cell line (Lx-2 cells) to study the susceptibility of human liver cells to PERV. We used rabbit anti-pig p15E polyclonal antibody against PERV to detect PERV protein.

**Materials and Methods**

**Cells and cell culture**

Phh cells were isolated from a 35-year-old woman who had heptectomy, using a 2-step perfusion technique with ethylenediaminetetraacetic acid and collagenase type II (Gibco, Grand Island, NY, USA); the liver was negative for human immunodeficiency virus, hepatitis B virus, and had no clinical evidence of cancer. The viability and number of isolated cells were quantified by the trypan blue (0.4%) exclusion test. The culture medium was complete Dulbecco modified eagle medium (Gibco) with 10% fetal bovine serum (catalog number 26140-079, Gibco), 1% penicillin (100 U/mL), and streptomycin (100 g/mL).

The HEK293 cells (ATCC, CRL-1573, WA, USA), Lx-2 cells (Institute of Regenerative Medicine, Southern Medical University, Guangzhou, China), HL-7702 cells (Chinese Academy of Sciences Committee Typical Culture Collection Cell Bank, Shanghai, China), and a porcine kidney cell line (PK15 cells) (ATCC, CCL-33, WA, USA) were purchased. All experimental protocols were approved by the Animal Care Ethics Committee and Clinical Investigation Committee, Southern Medical University; and conformed with the ethical guidelines of the 1975 Helsinki Declaration. Written, informed consent was obtained from all patients.

**Porcine endogenous retrovirus target cells**

The supernatant from a PK15 culture in exponential growth phase (cultured for 3 d) was collected and filtered using a 0.45-μm low-protein binding filter unit to remove cellular debris. The filtrate was added to culture plates that were seeded with HEK293, Lx-2, HL-7702, or Phh cells (1 × 10^5 cells) and cocultured for 24 hours. The supernatant was discarded, the plate was washed 3 times with phosphate buffered saline, fresh culture medium was added, and the culture was incubated for 1 week.

**Polymerase chain reaction and reverse transcription-polymerase chain reaction**

Cellular DNA was extracted using a kit (TIANamp Genomic DNA Kit, Beijing, China). The polymerase chain reaction conditions were as follows: pre-denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 95°C for 30 seconds; annealing at 58°C for 45 seconds; extension at 72°C for 1 minute; and final extension at 72°C for 7 minutes. After completion of the reaction, a sample (5 μL) of the polymerase chain reaction product was used for electrophoresis on a 2% agarose gel with a relative molecular weight marker (DL2000, Takara, Japan). The sequences of the primers used for the polymerase chain reaction were: PERV-pol (110 bp): forward 5’-ACAAAAACCCAGCAACAAG-3’ and reverse 5’-GAAGTCGGGACAGGTGATGT-3’; and p15E(417 bp): forward 5’-GGAATTCCGATGTCCAAAAGG-3’ and reverse 5’-CGGTCGACGTGAACTTTGCAGACATAT-3’.

Total RNA was extracted from the cells using a kit (Promega RNA Extraction Kit, Promega, Madison, WI, USA) and was stored at -80°C. The cDNA was synthesized using a reverse transcriptase (MMLV-RT, GenMag, EZ002-1, Beijing, China) according to the manufacturer’s instructions. The primers for reverse transcription polymerase chain reaction were synthesized commercially (Invitrogen, China), and amplification conditions were: 37°C for 15 minutes; 85°C for 5 seconds; 1 cycle of 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 90 seconds; and 1 cycle of 72°C for 7 minutes. The sequences of the PERV-pol (110 bp) primers were forward 5’-ACAAAAACCCCATCCACAAAG-3’ and reverse 5’-GAAGTCGGGACAGGTGATGT-3’. The pig transferrin receptor gene and human glyceraldehyde 3-phosphate dehydrogenase were used as internal standards; the corresponding primer sequences for pig transferrin receptor gene (80 bp) were forward 5’-GAGACAGAAACTTTCGAAGC-3’ and reverse 5’-GAAGTCTGTGATGCATCCAA-3’, and reverse 5’-GAAGTCTGTGATGCATCCAA-3’, and reverse 5’-GAAGTCTGTGATGCATCCAA-3’.
and the primer sequences for human glyceraldehyde 3-phosphate dehydrogenase (84 bp) were forward 5'-GGCAATCGTATTTTCGGCTTC-3' and reverse 5'-GTCCTATGCCTTAACAATCGC-3'.

**Rabbit anti-pig p15E polyclonal antibody**

For epitopes of PERV antigen p15E, E1(gpqqkglsl), E2(fegwlnrs), and its protein sequence, we cloned the p15E DNA from PK15 cells using polymerase chain reaction and successively expressed the p15E antigen protein in *E. coli* (BL21-Codonplus, Merck, Darmstadt, Germany) as described previously. The recombinant plasmids pGEX-4T-1/p15E were double digested by *EcoRI* and *BamHI*. The expressed protein was isolated from *E. coli* and was purified using a column chromatography filter layer (SP Sepharose Fast Flow Gel, Amersham Biosciences, Pittsburgh, PA, USA). The purified p15E antigen protein was used to immunize New Zealand rabbits. The titer and specificity of the antiserum were tested with enzyme linked immunosorbent assay and Western blot analysis.

**Western blot analysis**

The cells were lysed using radio immunoprecipitation assay lysis buffer (150 mM sodium chloride; 10 mM Tris [pH 7.2]; 0.1% sodium dodecyl sulfate; 1% Triton X-100; 1% deoxycholate; 5 mM ethylene-diaminetetraacetic acid; 1 mM phenylmethyl-sulfonyl fluoride; 10 mM benzamidine; 2 μg/mL leupeptin; 100 mM sodium orthovanadate; and 10 mM p-nitrophenyl phosphate), and the protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20 μL per well). The protein samples were transferred onto a nitrocellulose membrane and subjected to Western blot analysis using β-actin (Sigma, Shanghai, China) as the internal standard.

**Results**

The target fragment expressed in PERV-infected HEK293 cells was 110 bp, and no specific amplification appeared in the other cell types (Figure 1A). Expression of PERV was noted in PERV-infected HEK293 cells, and there was no PERV expressed in Phh, Lx-2, or HL-7702 cells (Figure 1B). The p15E DNA was amplified by polymerase chain reaction with an original length of 417 bp, and the amplified fragment was 417 bp (Figure 2A). There were bright bands both at 47 000 bp and 417 bp, which indicated that the recombinant plasmids pGEX-4T-1/p15E DNA were available (Figure 2B).

**Figure 1.** The PERV Expression of Different Cells by Polymerase Chain Reaction and Reverse Transcription-Polymerase Chain Reaction (M Marker)

**Figure 2.** Inducible Expression and Purification of p15E Antigen Protein

(A) Detection of PERV by polymerase chain reaction. Lane 1, positive control PK15 cells; lane 2, PERV-infected HEK293 cells; lane 3, HEK293 cells; lane 4, PERV-infected Lx-2 cells; lane 5, Lx-2 cells; lane 6, PERV-infected HL-7702 cells; lane 7, HL-7702 cells; lane 8, PERV-infected Phh cells; lane 9, Phh cells.

(B) Detection of PERV by reverse transcription-polymerase chain reaction. Lanes (1-9), internal control; lane 1', positive control PK15 cells; lane 2', PERV-infected HEK293 cells; lane 3', HEK293 cells; lane 4', PERV-infected Lx-2 cells; lane 5', Lx-2 cells; lane 6', PERV-infected HL-7702 cells; lane 7', HL-7702 cells; lane 8', PERV-infected Phh cells; lane 9', Phh cells.

(A) Amplification of p15E by polymerase chain reaction. M Marker DL2000; lane 1, p15E gene. (B) Determination of double-digested recombinant. M Marker DL15000 DNA; lanes 1 and 2, double digestion of recombinant; lanes 3 and 4, p15E gene. (C) SDS-PAGE of p15E recombinant protein. M Marker; lanes 1-5: samples of recombinant protein; 2 (D) results of purified p15E protein by SDS-PAGE. Lanes 1-5: Samples of purified p15E protein: M Marker.

The size of p15E protein was approximately 18 kD and the glutathione S-transferase tag protein was approximately 26 to 30 kD; therefore, the predicted size of the target fusion protein was 48 kD. After electrophoresis, there was a distinct protein band at 48 kD, confirming successful expression of the recombinant protein (Figure 2C). Purification of p15E protein by column chromatography filter layer was successful (Figure 2D).
The rabbit anti-pig p15E polyclonal antibody was identified by enzyme-linked immunosorbent assay and Western blot (Figure 3). Evaluation of p15E expression in PK15 cells with rabbit anti-pig p15E polyclonal antibody showed that a fragment appeared at the same level as p15E antigen protein at 18 kD, confirming specificity of the rabbit anti-pig p15E polyclonal antibody obtained (Figure 3A). The titer of rabbit anti-pig p15E polyclonal antibody was > 1:100,000 compared with the nonimmunization group (Figure 3B).

The PERV p15E was detected by Western blot using the rabbit anti-pig p15E polyclonal antibody. The PERV-infected HEK293 cells had the same fragment at 18 kD compared with the p15E antigen protein, but PERV-infected Phh, Lx-2, and HL-7702 cells did not express the PERV p15E protein (Figure 4).

Discussion

The potential risk of PERV infection limits the further application of porcine organs, tissues, and cells in xenotransplant and bioartificial liver in humans. However, at present, PERV cannot be completely eliminated, and it is expressed in different tissues and cells including porcine heart, liver, spleen, lung, kidney, islet, and vascular endothelial cells. The expression of viral mRNA may not be accompanied by the release of infectious particles, possibly because the majority of PERV loci contain frame shifts or stop codons, which may destroy ≥ 1 open reading frames. This potential risk of pathogen transmission is a key factor that limits the successful clinical application of porcine organs and cells. It is unknown whether PERV will become a clinical infection analogous to human immunodeficiency virus.

In this study, we evaluated 4 human cell types (cell lines and primary cells), including 3 types of human liver cells. The kidney cell line HEK293 was set as the control for sensitivity to PERV infection. The results suggested that 3 cell types from human liver were not infected by PERV. In contrast, HEK293 cells were highly susceptible to PERV infection, with expression of PERV in genomic DNA, RNA, and protein.

Furthermore, PERV may integrate into the genome but not be expressed because of a defective virus gene. Therefore, we studied both the integration of PERV into infected human cells, and also the expression of PERV RNA and protein in infected cells. The results showed that there was no integration of PERV or expression of PERV RNA or protein in 3 human liver cell types, suggesting that there were no significant changes in these human liver cells caused by PERV (Figures 1 and 4). This result cannot be generalized to all human liver cell types, and it is unknown whether PERV can infect the human liver in vivo because of the complexities of the human body and immune system and the activated state of PERV. Nevertheless, the present results show that not all human cells are susceptible to PERV in vitro.

In the present study, we produced rabbit anti-pig p15E polyclonal antibody. To increase the antigenicity and epitope integrity, we amplified p15E at 100 bp up-stream to express its protein sequence, and this showed that the antibody had a high titer and specificity. This antibody may be important in porcine xenotransplant and bioartificial liver procedures, and potentially may be useful in clinical detection of PERV. In conclusion, the human liver cells tested were not susceptible to PERV infection. Further studies may evaluate potential effects of PERV on human cells under different conditions in vitro and vivo.

Figure 3. Identification of Rabbit Anti-Pig p15E Polyclonal Antibody

(A) Western blot analysis of p15E antigen protein. M Marker; lanes 1-3: p15E antigen protein; lanes 4 and 5, PK15 cells; lanes 6 and 7, HEK293 cells.
(B) Identification of rabbit anti-pig p15E serum by ELISA.

Figure 4. Analysis of PERV by Western Blot

Lane 1, p15E antigen; lane 2, PERV-infected HEK293 cells; lane 3, HEK293 cells; lane 4, PERV-infected Lx-2 cells; lane 5, PERV-infected HL-7702 cells; lane 6, PERV-infected Phh cells.
References