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A study on the susceptibility of allogeneic human hepatocytes to porcine endogenous retrovirus

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Abstract. – OBJECTIVE: Porcine endogenous retrovirus (PERV) is a virus that can be integrated into porcine genome. It has been proved that PERV can infect the cells of a variety of species. However, little is known about the infectivity of PERV to human hepatocytes. The present study focused on the susceptibility of primary human hepatocytes to PERV.

MATERIALS AND METHODS: Cell culture supernatant containing PERV was used to infect primary allogeneic hepatocytes and human embryonic kidney cell line HEK-293. The integration of PERV into the genome and PERV expression were detected by using PCR and RT-PCR.

RESULTS: Gene and mRNA sequences of PERV were detected in HEK-293 cells; however, viral gene expression was not detected in 3 groups of primary allogeneic hepatocytes.

CONCLUSIONS: HEK-293 cells can be infected by PERV, but 3 groups of primary allogeneic hepatocytes were not sensitive to PERV, indicating PERV had no infectivity to primary human hepatocytes.

Key Words:
Bioartificial liver, Porcine, Porcine endogenous retrovirus (PERV), Infection, Hepatocytes.

Introduction

Shortage of allogeneic donor organs is one of the major challenges of organ transplantation. Rapid technological development has already made xenotransplantation possible[1]. Pigs are a good source of donor organs for xenotransplantation because of similarities in anatomy and physiological functions with human, low cost, short breeding cycle and ease of breeding transgenic animals[2-5]. However, two problems need to be solved clinically. Firstly, hyperacute immunological reaction may be caused by different immune background. It can be solved by Gal knockout or intercellular transfer of complement regulatory factors. Secondly, biosafety problems may be induced by pathogenic microbes in pigs. Infections by some microbes may be avoided by scientific breeding and management[6]. However, some other microbes, such as PERV, are hardly removed from the donor organs, since PERV is usually integrated into porcine genome[7]. Primary endotheliocytes, hepatocytes, lung cells and skin cells of different species of pigs all express PERV, leading to zoonoses. It was discovered in 1971 that a retrovirus-like particle produced by pig kidney cell line PK-15 did not cause infections in pigs. No more studies on the infectivity of this retrovirus-like particle were done after that. Patience et al[8] reported in 1997 that this retrovirus-like particle produced by pig kidney cell line PK-15 could infect HEK-293, and the infectivity of PERV has caught extensive attention since then. Wilson et al[9] have shown that normal pigs may produce viral particles that are infectious to human. Luc et al[10] demonstrated for the first time that PERV was able to cause cross-species infection *in vivo*. Having proved that pig islet cells could produce PERV that was infectious to human cells, Luc et al[10] transplanted pig islets into NOD/SCID mice (mice with non-obese diabetes mellitus and severe combined immunodeficiency). Viral gene expression was detected in the spleen, liver, kidney and small intestines. Many experiments have shown that PERV can infect a variety of primary cells and cell lines *in vitro* and *in vivo*. Of three subtypes of PERV, PERV-A and PERV-B can infect cells of different species, but PERV-C only infects swine-derived cells. Specke et al[11] found that porcine peripheral blood monocytes and lung epithelial cells of mink infected human cells in co-culture. Under the electron microscope, the mink cells infected by PERV presented with multivascular bodies containing C-type retroviruses and small virus-like particles;

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but those not infected by PERV had no such multivascular bodies. PERV infection in interstitial spaces after transplantation of pig islet cells into SCID mice was observed in 2000, which suggested the infectivity of PERV *in vivo*[12]. Deng et al[13] showed that PERV was integrated into the genome of liver and spleen of SCID mice after transplantation of embryonic pig pancreatic cells. Studies on the *in vitro* infectivity of PERV derived from different tissues of pigs can provide reference for transplantation of pig-derived organs. However, only a few studies are devoted to the infectivity of PERV to hepatocytes. We discussed the infectivity of PERV to allogeneic human hepatocytes, with the focus laid on interindividual differences in terms of sensitivity to PERV.

Materials and Methods

Cells and cell lines: primary human hepatocytes were harvested from liver tissues by two-step infusion method and stained by Trypan blue (0.4%). After cell viability evaluation and cell count, the cells were cultured with 10% fetal bovine serum (FBS) and Williams medium containing 1% double antibodies. HEK-293 cells (ATCC, CRL-1573, Washington, DC, USA) were cultured with DMEM containing 10% fetal bovine serum (FBS) and 1% double antibodies. Cell passage was performed once every 5-6 days.

PERV Infection of Target Cells

Viral supernatant was collected three days after passage of PK15 cells. Cell fragments were removed by using a 0.45 μm filter. Into each culture flask 5 ml of cell culture solution was added to infect the cells at 37 for 24h. Viral liquid was replaced after 24h by fresh culture medium. PERV-infected cells were harvested 1 week later.

DNA Extraction and RNA Isolation

Genome DNA Purification Kit (Promega, Madison, WI, USA) was used for DNA extraction according to the manufacturer's instruction; RNA extraction was performed using Total RNA Purification Kit (Promega, Madison, WI, USA). Primers GF2/GR2 were localized to pol gene of PERV; upstream primer was 5-CCACAGGGCAACGACAGTATCCATG-3, and downstream primer was 5-TTGGAGGGTCAACACAGTGATCGG-3; primers TF1/TR1 were localized to gag gene of PERV; upstream primer was 5-CGGCAAGAGAAGAATTTGACTAAGATC-3, and downstream primer was 5-CAGTTCCTTGCCCAGTGTCCTCTT-3.

RNA Purification and Integrity

RNA purification system was prepared by using RNASE-free DNASE I (Promega), followed by digestion at 37 for 30 min and deactivation at 65 for 10 min. Equal volume of phenol was added and mixed by turning the flask upside down. Cells were centrifuged at 10000 rpm for 5 min, and the supernatant was collected. The procedures were repeated twice and equal volume of isopropyl alcohol was added and gently mixed. After the solution stood at -20 for 15 min, the cells were centrifuged at 10000 g at 4 for 10 min, and the supernatant was discarded. Precipitate was collected, washed twice with 75% ethanol and air dried on a super clean bench. The precipitate was finally dissolved by adding 15-40 μL diethylpyrocarbonate (DEPC) water.

Fifty-fold dilution was performed for 1 μL RNA. OD value was measured with the BioPhotometer plus (Eppendorf, Hamburg, Germany).

After 1% agarose gel electrophoresis using 1 μL RNA at 80V for 20 min, the bands of 5s rRNA, 18s rRNA and 28s rRNA were observed with a gel imaging system.

Reverse Transcription of RNA

Reverse transcription system was prepared in an RNase free PCR tube with gentle blowing. The solution was kept at 85 for 5 min and then cooled immediately on ice. After addition of Oligo (dT), random primer, 10 mM dNTP, RNase inhibitor, 5 × buffer and M-MLV, the solution was kept at 30 for 10 min, 42 for 60 min and 85 for 10 min, successively.

PCR and RT-PCR

Genomic DNA and total RNA extracted from PERV-infected HEK-293 cells and allogeneic primary hepatocytes were used as templates; genomic DNA and total RNA from uninfected cells were used as negative control, and those from PK15 cells as positive control. HEMA9600 PCR Thermo Cycler was used for PCR amplification: denaturation at 95 for 5 min, 94 for 1 min, 57 for 1 min, 72 for 1 min, 35 cycles, final extension at 72 for 6 min; RT-PCR amplification: denaturation at 95 for 5 min, 94 for 1 min, 57 for 1 min, 72 for 1 min, 35 cycles, final extension at 72 for 6 min. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (571 bp) was used as

Table I. PERV infection of allogeneic primary hepatocytes and HEK-293 cells.

Sample	DNA PCR	RNA RT- PCR
PK15	+	+
HEK-293	–	–
HEK-293+PERV	+	+
Group A of primary human hepatocytes	–	–
Group A of primary human hepatocytes+PERV	–	–
Group B of primary human hepatocytes	–	–
Group B of primary human hepatocytes+PERV	–	–
Group C of primary human hepatocytes	–	–
Group C of primary human hepatocytes+PERV	–	–

–: No DNA or RNA of PERV was detected. +: No DNA or RNA was detected.

internal control. The amplified products were analyzed along with markers (100 bp, 250 bp, 500 bp, 750 bp, 1000 bp, 2000 bp) by using 2% agarose gel electrophoresis. Images were taken after EB staining. The relative DNA and mRNA abundance was evaluated by gray value.

Statistical Analysis

All data were expressed as mean ±SD. Results were analyzed by one-way ANOVA, and Student's *t*-test was used for judging statistical significance if differences were established. $p < 0.05$ was considered statistically significant.

Results

PERV Infection of HEK-293 Cells

It is indicated by a large number of experiments that PERV can infect HEK-293 cells with high sensitivity. Proviral copy number and expression of PERV were detected by using PCR and RT-PCR (Table I). Gap gene (187 bp) and pol gene (212 bp) were integrated into the genome of HEK-293 cells according to the results (Figure 1A), along with mRNA expressions of related genes (Figure 1B). Thus PERV was capable of infecting HEK-293 cells. To prevent false-positive result, genomic DNA of PK15 cells and the products expressed by DNA were taken as positive control. Results showed that the copy numbers of gap and pol genes were high in the genome of PK15 cells and gap and pol genes were expressed.

PERV Infection of Allogeneic Primary Hepatocytes

For experimental group, primary human hepatocytes were co-cultured with supernatant containing PERV; for negative control, the primary human hepatocytes were not infected by PERV. No pol or gap gene was detected in the genome of allogeneic primary hepatocytes; no specific bands were amplified in the negative control, neither (Figure 2A, C, $p < 0.001$). No mRNA expressions of pol and gap genes were found using RT-PCR (Figure 2B, D $p < 0.001$). To prevent

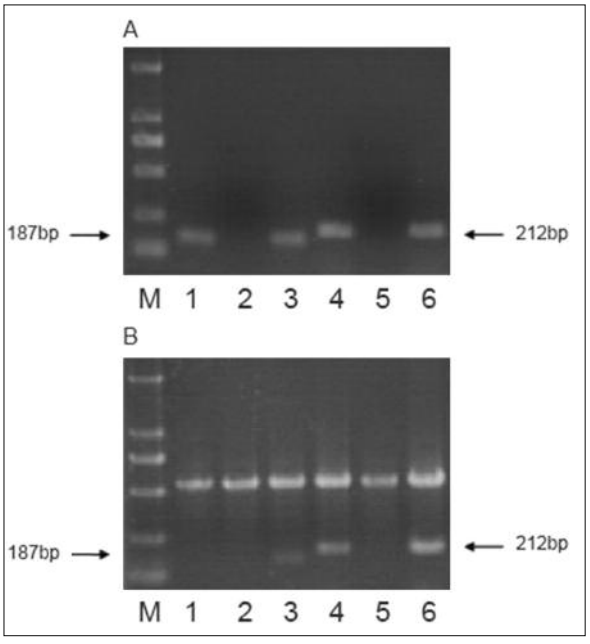

Figure 1. ***A,*** The integration of PERV genes (pol-212bp, gap-187bp) into the genome of HEK-293 cells was analyzed by using PCR after co-culture of HEK-293 cells with supernatant containing PERV. ***B,*** Expression of PERV genes (pol-212bp, gap-187bp) in HEK-293 cells after co-culture of HEK-293 cells with supernatant containing PERV was analyzed by RT-PCR. Results showed: Lane1 PK15, Lane 2 HEK-293, Lane3 HEK-293+PERV, Lane4 PK15, Lane5 HEK-293, Lane6 HEK-293+PERV. M represents marker, with the length of 100, 250, 500, 750, 1000 and 2000 bp from bottom to top.

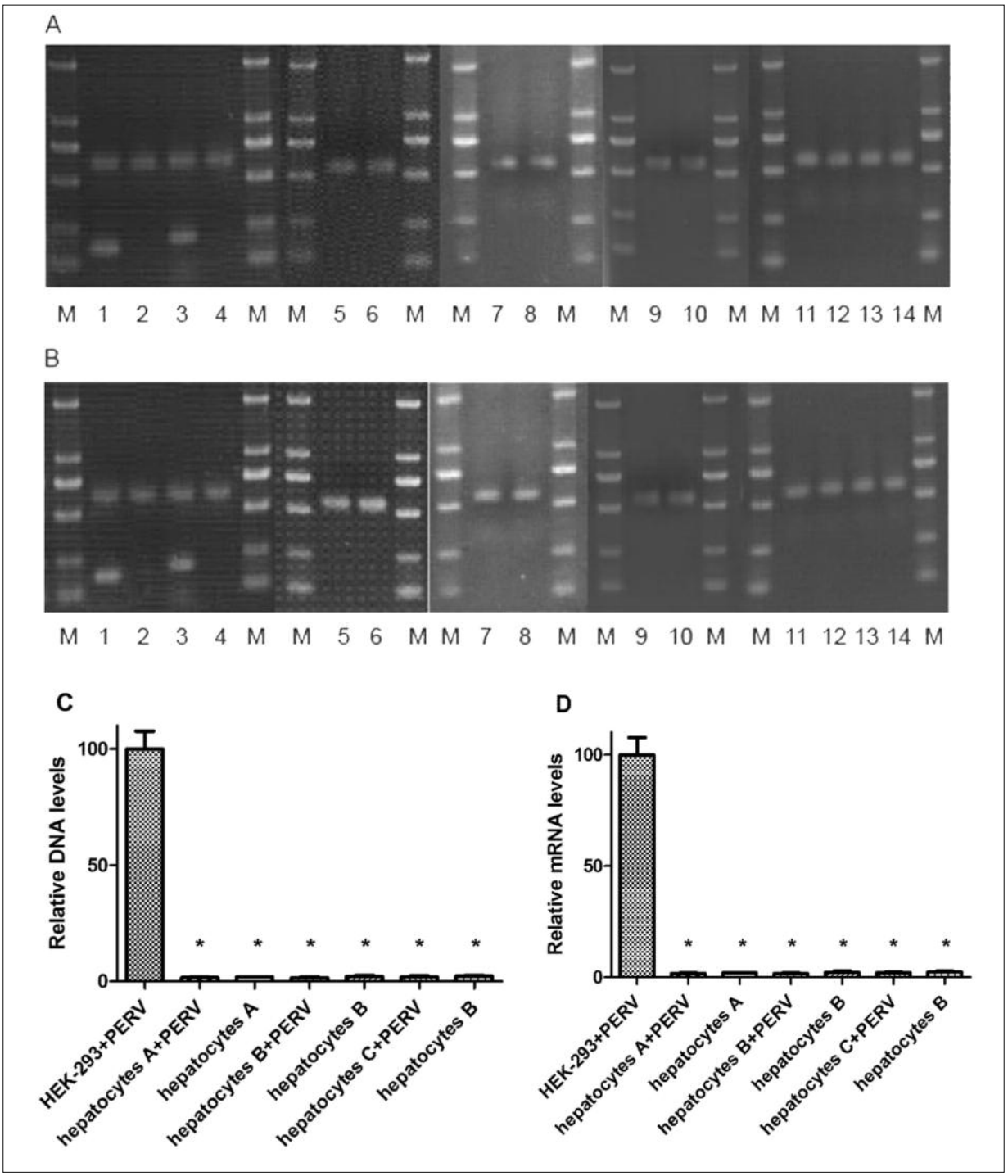

Figure 2. A, Integration of PERV genes (pol-212bp, gap-187bp) into the genome of primary human hepatocytes was analyzed by using PCR after co-culture with supernatant containing PERV. ***B,*** Expressions of PERV genes (pol-212bp, gap-187bp) in primary human hepatocytes were detected by using RT-PCR after co-culture with supernatant containing PERV. C, D: Histogram of relative DNA and mRNA abundance in different groups. Lane1 HEK-293+PERV, Lane2 Group A of human hepatocytes +PERV, Lane3 HEK-293+PERV, Lane4 Group A of human hepatocytes+PERV, Lane5 control treatment for group A of human hepatocytes+PERV, Lane6 control treatment for group A of human hepatocytes, Lane7 group B of human hepatocytes+PERV, Lane8 group B of human hepatocytes +PERV, Lane9 control treatment for group B of human hepatocytes, Lane10 control treatment for group B of human hepatocytes, Lane11 group C of human hepatocytes+PERV, Lane12 group C of human hepatocytes+PERV, Lane13 group C of human hepatocytes, Lane14 control treatment for group C of human hepatocytes. (*$p < 0.001$ compared with HEK-293+PERV group, n = 3).

Tests of between-subjects effects. Dependent variable: gene number.

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	.000[a]	2	.000	.	.
intercept	.000	1	.000	.	.
Infect factor	.000	1	.000	.	.
group	.000	1	.000	.	.
Infect factor*group	.000	1	.000		
Error	.000	3	.000		
Total	.000	6			
Corrected Total	.000	5			

[a]R squared = . (Adjusted R Squared = .)

false-positive result caused by DNA pollution, DNA digestion with DNaseI was performed during RNA isolation; RNA purity and integrity were analyzed. OD260/OD280 ratio above 1.8 indicated that the isolated RNA was pure and not polluted by proteins. After agarose gel electrophoresis for the isolated RNA, the bands of 5s rRNA, 18s rRNA and 28s rRNA were observed with the gel imaging system. All three bands were intact, indicating complete total RNA isolation process.

Discussion

Xenotransplantation is a promising approach to solve the shortage of donor organs. Since the first discovery of PERV in pigs by Patience et al[8] in 1997, increasing attention has been drawn to the cross-species transmission of xenogeneic animal diseases through transplantation of pig organs, tissues and cells. This is the major barrier to clinical application of xenotransplantation and bioartificial liver[14,15]. PERV genes can be integrated into porcine genome and passed down to offspring according to Mendelian law of inheritance. Only a small part of 30-50 PERV proviral sequences present in porcine genome express infectious viral particles[16]. Some authors[17,18] reported that PERV may infect some human-derived cell lines and primary cells *in vitro*, with PERV proviral sequences detected in the genome of the infected cells. However, various retrospective clinical trials carried out by foreign researchers[10] did not find evidence of PERV infection in cases transplanted with live pig tissues and hemodialysis cases receiving pig kidney transplantation. All these point to the risk of cross-species transmission of PERV through xenotransplantation in theory, though the actual risk of cross-species transmission of PERV is small. Whether PERV will induce infection in human after xenotransplantation and spread among population is still a disputable topic. Retroviral infection is an integration process of proviral DNA sequences into the genome of hosts. We detected the proviral PERV sequences and PERV expression in hosts, so as to assess the risk of cross-species transmission. Considering the occurrence of gene deletion during proviral infection of target cells, PCR amplification was performed using primers located in the gag and pol genes. The results showed that there was no gene deletion in PERV-infected HEK-293 cells and the functional genes were integrated intact. According to some studies, PERV cannot infect primary human hepatocytes[19]. We verified the infectivity of PERV to primary human hepatocytes using allogeneic hepatocytes from 3 sources. No proviral PERV sequences were detected in the genome of hosts, indicating that PERV was not capable of infecting primary human hepatocytes, or at least not stably. RT-PCR detection revealed that viral genome was not expressed in host cells, suggesting incapability of PERV genes to be integrated into the genome of primary human hepatocytes.

Conclusions

The infectivity experiments in the present study confirmed that PERV was not able to infect allogeneic human hepatocytes. The infectivity of PERV to human immune system is related to various factors. The potential risk of cross-species transmission of PERV deserves our full attention before the explicit conclusion is drawn from *in vivo* experiments.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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