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Studies on long-term infection of human cells with Porcine endogenous retrovirus

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Summary. – A major concern in pig-to-human xenotransplantations is the potential risk of transmission of Porcine endogenous retroviruses (PERVs) integrated in the pig genome. Our previous work has shown that PERV provirus genes and gag protein can be detected in human embryonic kidney HEK-293 cells during a long-term infection with PERV (Yu *et al*., *Transplant. Proc*. **37**, 496–499, 2005). In this study, we continued studying the long-term (>6 months) PERV infection of HEK-293 cells. The results showed no significant differences in morphology, growth, apoptosis, and [^{3}H]-thymidine incorporation between PERV-infected and uninfected cells. The PERV LTR sequence showed only an insignifcant mutation after the long-term infection. PERV infection had no effect on the transcription of genes of Human endogenous retrovirus (HERV) naturally occurring in HEK-293 cells. Summing up, this study indicated that a long-term PERV infection of HEK-293 cells *in vitro* does not result in any significant changes in host cells as well as in PERV LTR sequence.

Keywords: HEK-293 cells; Human endogenous retrovirus K; Porcine endogenous retrovirus

Introduction

In light of the severe shortage of human donor organs for transplantation procedures, pig-to-human xenotransplantation provides an effective alternative for treating various intractable diseases and the end-stage organ failures (Dinsmore *et al.*, 2000; Heneine *et al.*, 2001; Van De Kerkhove *et al.*, 2003). However, the discovery of *in vitro* infection of human cells with PERVs has raised zoonosis-associated biosafety concerns, which have become the primary obstacle for the use of this novel therapeutic modality (Martina *et al*., 2003; McIntyre *et al.*, 2005). PERV is a major pathogen that can be possibly transmitted to human beings, and many experiments have been conducted to evaluate the potential risk of cross-species transmission of PERV; however, there is no clear evidence of the ability of PERV to transmit to human beings and cause symptomatic diseases.

Our previous study showed that PERV *gag*-pol gene and gag protein were present in HEK-293 cells after a 6-month infection and these cells exhibited the same DNA content as uninfected cells (Yu *et al.*, 2005). In this study, using the same system, i.e. the long-term (>6 months) infection of HEK-293 cells with PERV, we investigated morphology and growth of the host cells and expresion of gag protein of the virus. Since the long terminal repeat (LTR) region of PERV genome is a dynamic region responsible for virus integration and replication (Kuddus *et al.*, 2003), we also determined the mutation rate of PERV LTR. Eventually, since PERV infection may activate HERV, which is regarded as associated with the development of germ-cell tumors, we also studied transcription of HERV genes in PERV-infected HEK-293 cells (Kuddus *et al*., 2003).

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Abbreviations: PERV = Porcine endogenous retrovirus; HERV = Human endogenous retrovirus

Materials and Methods

Cells and virus. Pig kidney cell line PK15 and human embryonic kidney cell line HEK-293 were employed. Culturing of the cells and preparation of PPRV from PK15 cells were described previously (Yu *et al*., 2005).

Electron microscopy. To examine the morphology of PERV the supernatant of PPRV-infected HEK-293 cells at the 63rd passage was harvested, concentrated using a rabbit PERV gag-antibody according to Krach *et al.* (2000), and subjected to electron microscopy in standard way

PCR for PERV LTR. Total DNA was extracted from cells using a blood genomic DNA extraction kit (Pharmacia, USA). PERV LTR-specific PCR was caried out using the primers described earlier (Kuddus *et al.*, 2003) and the following conditions: 35 cycles of 95°C/5 mins, 94°C/1 min, 60°C/1 min, and 72°C/1 min, and final extension at 72°C for 9 mins.

Cloning and sequencing of PERV-LTR was done as described previously (Zhang *et al.*, 2006).

Western blot analysis. To assay PERV gag protein a rabbit PERV gag-antibody (1:400 dilution) was employed as primary one. For optimal semiquantitative analysis of the blots equal numbers of cells and equal volumes of protein samples were used. The assay was done in standard way (*Ye et al*., 2006).

MTT assay. To assay cell growth, 5 x 10^3 cells/well were plated in 96-well plates, cultivated and subjected to standard MTT assay, in which A_{570} was determined (*Ye et al*., 2006).

[^{3}H]-thymidine incorporation. 5 x 10^3 cells/well were plated in 96-well plates and, after 24 hrs of cultivation, 1.0 μCi of [methyl-^{3}H] thymidine (Sigma, USA) per well was added. After further 24 hrs, the incorporated radioactivity was measured using a scintillation counter (*Ye et al*., 2006).

Flow cytometry. To assay apoptosis propidium iodide staining and flow cytometry were employed. The staining consisted of incubating equal volumes of propidium iodide and cell suspension for 30 mins.

RT-PCRs for HERV-K gag and env mRNAs. Total RNA was extracted from cells by using an RNA extraction kit (Promega, USA). RT-PCRs specific to HERV-K gag and env mRNAs were done using specific primers described earlier (Kuddus *et al.*, 2003). The following conditions were employed: incubations at 50°C for 30 mins, at 94°C for 2 mins, and at 95°C for 5 mins, respectively, 35 cycles of 94°C/1 min, 66°C (gag) or 58°C (env) /1 min, and 72°C/1 min, and final extension at 72°C for 6 mins. GAPDH mRNA served as internal standard. RT-PCR products were analyzed by agarose gel electrpophoresis.

Statistical analysis. The obtained results were expressed as means ± SE. Statistical analysis was performed using the *Student's t*-test. Differences with $P \leq 0.05$ were considered significant.

Results

Morphology of PERV

The PERV particles prepared from the supernatant of PERV-infected HEK-293 cells exhibited a typical retroviral morphology, namely a round shape of approximately 120 nm in diameter and a dense core and capsid (Fig. 1).

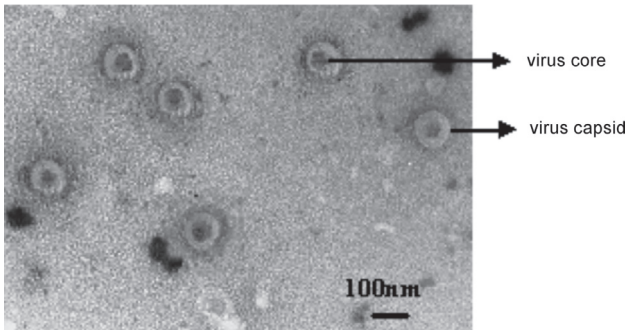

Fig. 1

Electron microscopy of PERV

The 63rd passage of PERV-infected HEK-297 cellls was used for preprartion of the virus. Magnification 42,000x.

Mutational rate of PERV LTR

The PERV-LTR region was successfully cloned from the PERV-infected HEK-293 cells and the obtained clone was sequenced. A comparison of this sequence with corresponding ones available in GenBank (AY099323 and AY099324) revealed a low rate of mutation of PERV LTR in PERV-infected HEK-293 cells, namely from 3 x 10^{-5}/bp to 1 x 10^{-4}/bp per generation.

PERV gag protein expression in PERV-infected HEK-293 cells

Western blot analysis of PERV-infected HEK-293 cells showed a strong signal at 60 K, a weak signal at 40 K, and no signal at 10 K corresponding to various forms of PERV gag protein, respectively. PK15 cells yielded similar results, while uninfected HEK-293 cells were entirely negative (Fig. 2).

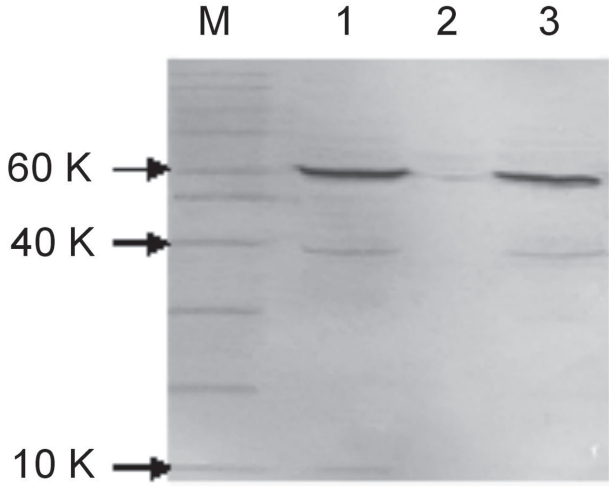

Fig. 2

Western blot analysis specific for PERV gag protein

Protein ladder (lane M), PK15 cells (lane 1), uninfected (lane 2), and PERV-infected HEK-293 cells (lane 3).

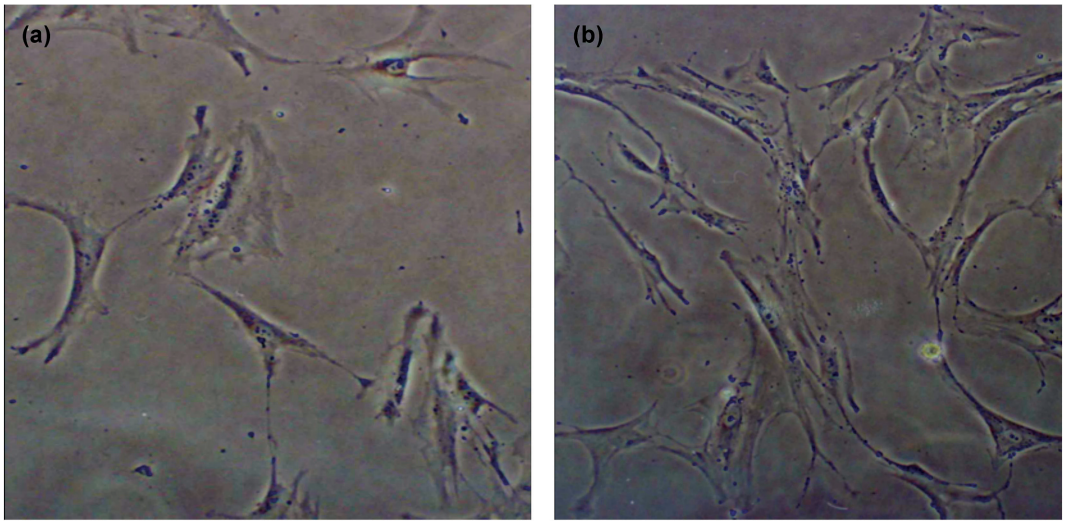

Fig. 3

Morphology of uninfected (a) and PERV-infected (b) HEK-293 cells

Magnification 400x.

Morphology of PERV-infected HEK-293 cells

Morphology of PERV-infected HEK-293 cells as revealed by light microscopy was similar to that of the uninfected control cells, namely spindle-shaped or polygonal (Fig. 3).

Growth, [^{3}H]-thymidine incorporation, and apoptosis in PERV-infected HEK-293 cells

A comparison of the growth of PERV-infected and uninfected HEK-293 cells by MTT assay did not reveal any significant difference (Fig. 4). A similar result was obtained by using [^{3}H]-thymidine incorporation as measure of cell proliferative activity. Again, the values of ^{3}H counts/cell for both series did not differ significantly (0.21 ± 0.03 vs. 0.19 ± 0.02) (Fig. 5).

Finally, apoptotic rates of PERV-infected and uninfected HEK-293 cells were determined by flow cytometry (Fig. 6). Also in this case similar result was obtained, the difference was insignificant (9.8 ± 3.3 vs. 9.5 ± 2.8).

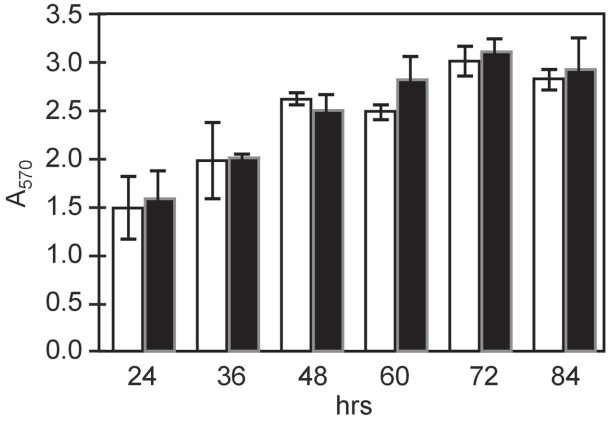

Fig. 4

Growth of uninfected and PERV-infected HEK-293 cells

MTT assay was performed at 24–84 hrs post seeding of cells. Uninfected (empty columns) and PERV-infected HEK-293 cells (full columns).

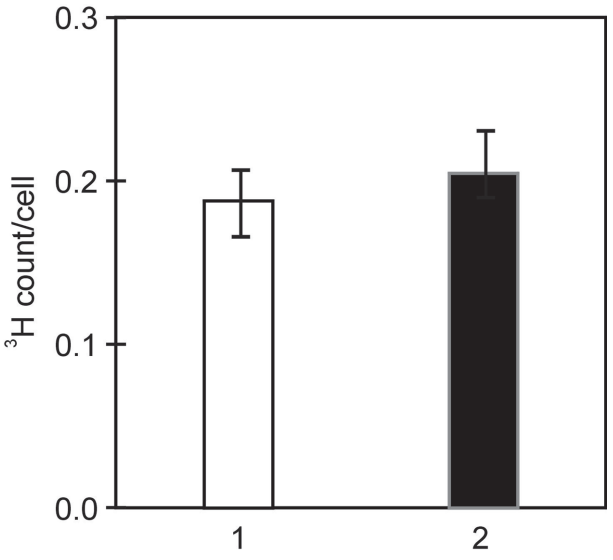

Fig. 5

[^{3}H]-thymidine incorporation in uninfected (1) and PERV-infected (2) HEK-293 cells

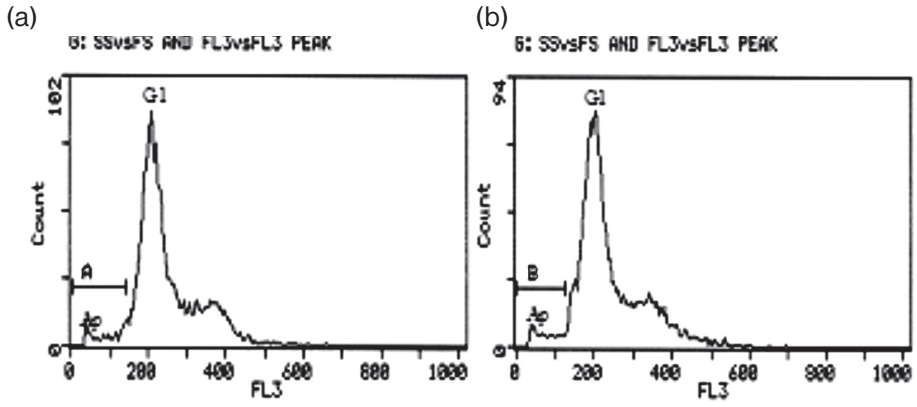

Fig. 6

Apoptosis in uninfected (a) and PERV-infected (b) HEK-293 cells

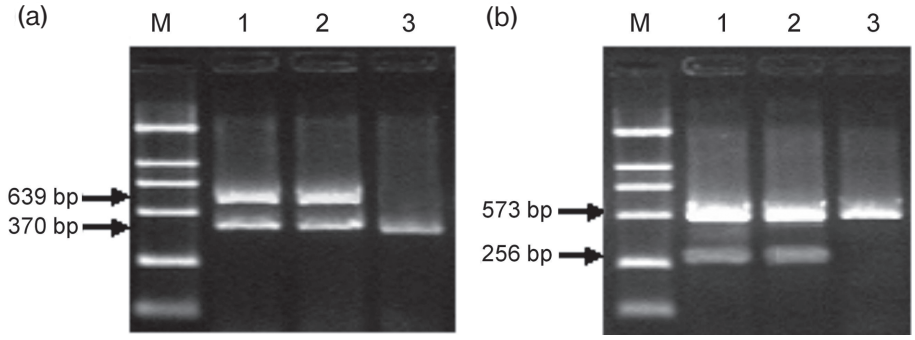

Fig. 7

RT-PCR detection of HERV-K gag (a) and env (b) mRNAs in uninfected and PERV-infected HEK-293 cells

Agarose gel electrophoresis of RT-PCR products. Size markers (lane M), uninfected (lane 1) and PERV-infected (lane 2), HEK-293 cells, PK15 cells (lane 3). The bands of 370 bp (A) and 573 bp (B) correspond to GAPDH mRNA.

Transcription of HERV-K genes in PERV-infected HEK-293 cells

To test the possible activation of HERV by PERV infection the transcription of the HERV-K *gag* and *env* genes in PERV-infected and uninfected HEK-293 cells was examined by Western blot analysis (Fig. 7). Both gag mRNA (the 639 bp band) and env mRNA (the 256 bp band) were detected in both cell series and a semiquantitative analysis showed no significant differences between their levels. PK15 cells as control gave negative result for both mRNAs.

Discussion

A potential xenozoonosis associated with pig-to-human xenotransplantation, especially the possible risk of PERV transmission, has become a matter of concern and a major barrier for xenotransplantation (Sypniewski *et al*., 2005). In this study, we exposed human HEK-297 cells to PERV and passaged the infected cells for an extended time period. To prove the production of PERV particles by these cells, we subjected the supernatant from the 63rd passage of such a culture to electron microscopy. We observed particles with a dense core and capsid, showing a typical C-type retroviral morphology.

PERV as a retrovirus may undergo high-rate genomic rearrangements including point mutations, deletions, duplications, inversions, and any of their combinations (Coffin, 1990). PERV LTR is a region that is assumed to exert a significant influence on PERV replication and pathogenicity. Therefore, in this study, we investigated possible changes in the LTR sequence during long-term *in vitro* culturing of PERV-infected HEK-297 cells. The sequence analysis revealed only a low mutational rate (from 3 x 10^{-5}/bp to 1 x 10^{-4}/bp per generation) in the infected HEK-293 cells. Considering

the mutational rate of thermostable DNA polymerase of 8.9 x 10^{-5}/bp and taking into account a background mutational rate during retrovirus replication and transcription of approx. 1 x 10^{-4}/bp, we can deduce that the mutation of the PERV LTR during the long-term infection of HEK-297 cells with PERV did not have any apparent significance.

It was unclear whether PERV could transmit to humans and cause symptomatic diseases, necessitating studies on the effects of long-term PERV infection on human cells. The results of the analysis of cell morphology, growth, cell proliferation and apoptosis (data shown here), and DNA content/cell (data reported previously) indicated that there were no growth abnormalities in PERV-infected HEK-293 cells. The Western blot analysis specific for PERV gag protein showed in PERV-infected HEK-297 cells a strong signal for the 60 K precursor and a weaker one for the 40 K intermediate. In comparison, PK15 cells exhibited an additional 10 K protein. This result may indicate that the variety of forms of PERV gag protein in PERV-infected HEK-293 cells was reduced after the long-term infection. The undetectable 10 K PERV gag protein in infected human cells is subject of further studies.

Endogenous retroviral HERV sequences constitute about 1% of the human genome. Most members of the HERV family in the human genome are defective and only a few members of the HERV-K family are nearly intact. These intact members can be detected in some normal tissues, tumors and transformed cell lines. HERV genes have been shown to be associated with germ cell tumors, breast cancer and some leukemias and their sequences have some similarities to PERV (Herbst *et al.*, 1996). Since retroviral genomes may exhibit high rates of recombination when target cells are coinfected with two related or unrelated exogenous and endogenous retroviruses, a PERV infection may affect the transcription of HERV-K. In this study, HERV-K transcripts were detected in PERV-infected and uninfected HEK-293 cells in similar levels in accord with the data reported previously (Kuddus *et al.*, 2003). However, the effects of PERV infection on other HERV families have not been investigated yet.

Although a number of experiments have proven that PERV can infect human cells *in vitro*, it is not known whether PERV can infect them *in vivo* (Ritzhaupt *et al.*, 2002; McIntyre *et al.*, 2003; Martina *et al.*, 2005). Many studies have indicated that the risks of cross-species transmission of PERV for immunocompetent individuals and allotransplant recipients are negligible, even if these individuals are in close and repeated contact with live pigs or pig tissues. Moreover, there is no evidence of PERV infection in health care workers exposed to transgenic porcine liver extracorporeal support (Levy *et al.,* 2000, 2007; Sauer *et al.,* 2003; Irgang *et al.,* 2003; Hermida-Prieto *et al.,* 2007). Our study indicates that a possible PERV infection during xenotransplantation would not induce acute and severe pathological changes in the patients. Since we restricted our studies to some specific growth characteristics of PERV-infected human cells, we think that further studies should focus on the effects of PERV infection on the biology of human cells in general and on their genome in particular.

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