TRANSMISSION OF PORCINE ENDOGENOUS RETROVIRUS TO HUMAN CELLS IN NUDE MOUSE

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Summary. – Xenotransplantation is associated with the risk of Porcine endogenous retrovirus (PERV) transmission, since it has been shown that PERV can infect human cells *in vitro* (Specke *et al*., *Virology* **285**, 177–180, 2001). We evaluated the possibility of PERV infection of human cells in nude mice model. Porcine kidney cells PK15 carrying PERV and human liver cancer cells SMMC-7721 were injected separately into the right and left axilla of nude mice, respectively. Two months later, pig cytochrome oxidase II (COII) gene, PERV DNA, PERV mRNA, and PERV-Gag protein were detected in the mass formed in both axillas and in several organs of nude mice. The pig COII genes were detected in the right and left axilla, but not in other organs of nude mice implicating that the microchimerism of pig cells occurred in human SMMC-7721 cells and induced the formation of the mass. PERV gene and *gag* protein were detected in all mouse tissues except liver. These data indicated that (i) PERV may be transmitted from porcine to mouse cells, (ii) PERV genes and proteins were detectable in the mass formed by injection of human cells and consequently (iii) there was a possibility of PERV transmission to human cells after xenotransplantation.

Key words: Porcine endogenous retrovirus; xenotranplantation; transmission *in vivo*

Introduction

Transplantation of pig organs to humans is prospective, but there is a concern about the biohazard possibly brought by the transmission of PERV that is stably integrated into the genome of pigs and cannot be removed by current methods (Smetanka and Cooper, 2005; Boneva and Folks, 2004; Dorling, 2002). PERV have been classified into the retroviral ß (B- or D-type) and γ (C-type) genera (van Regenmortel *et al*., 2000; Tidona and Darai, 2001). Infectious human tropic PERV have been assigned to the PERV γ1 family consisting of the subfamilies A, B and C (Takeuchi *et al*., 1998; Patience *et al*., 2001). PERV particles can be produced by porcine cell lines, tumor cells, aorta endogenous cells, and peripheral blood mononuclear cells (Wilson *et al*., 1998). Previous study has shown that pig kidney cell line PK15 spontaneously release PERV particles, which can infect human primary cells and cell lines *in vitro* (Specke *et al*., 2001).

In this study, the infectivity of PERV *in vivo* was evaluated by establishing a nude mouse model of cell transplantation. The transmission of PERV from PK15 cells to the transplanted human cells and to the organs of experimental mouse was examined.

Materials and Methods

Cell culture. PERV-producing cell line, PK15 was obtained from ATCC (CCL-33). Human liver cancer cell line, SMMC-7721 used as target cells was from Cell Biology Institute of Shanghai (Chinese Academy of Sciences). Both cell lines were maintained in DH (Dulbecco's Modified Eagle Medium with high glucose) medium containing 10% fetal bovine serum.

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Abbreviations: COII = cytochrome oxidase II; PERV = Porcine endogenous retrovirus

Transplantation of PK15 and SMMC-7721 cells to nude mice. Six to eight weeks old nude mice (n = 6) were obtained from Experimental Animal Center (West China Medical Center, China). Altogether, 1×10^7 PK15 and 5×10^6 SMMC-7721 cells were injected subcutaneously in the right and left axilla of the nude mice, respectively. The mice grafted with SMMC-7721 cells only in the left axilla were used as the negative control. Mice were monitored up to 6–8 weeks post transplantation.

PCR of pig COII gene. DNA and total RNA were extracted from mice organs including liver, kidneys, lungs, heart, and the mass in the right and left axilla using Dneasy Tissue Kit (Qiagen) and SV Total RNA Isolation System (Promega). To detect the microchimerism of PK15 cells and exclude the contamination of pig cells in the tissues of nude mice, pig mitochondrion COII gene was examined. The PCR system contained 1×PCR buffer, 200 μmol/l dNTPs, 2 U of rTaq DNA polymerase (TaKaRa), 15 pmol of each primer, template, and distilled H_2O to a final volume of 25 μl. Primers for amplification of COII gene were COII-forward (5'-CATTGGAGTAGTCCTACTATTTACCG-3') and COII-reverse (5'-GTAGGATTAGTATTATAAAT AAGGCTCCT-3'). The PCR profile was one cycle of 96°C for 2 mins, 35 cycles of 95°C for 30 secs, 55°C for 30 secs, 72°C for 3 mins, and final extension of 72°C for 5 mins. PCR products were analyzed by 2% polyacrylamide gel electrophoresis.

PCR of PERV DNA. PCR for amplification of *gag* and *pol* genes was performed with the following primers: *gag*-forward (5'-CGGCAAGAGAAGAATTTGACTAAGATC-3') and *gag*-reverse (5'-CAGTTCCTTGCCCAGT GTCCTCTT-3'); *pol*-forward (5'-CCACAGGGCAACGACAGTATCCATG-3') and *pol*-reverse (5'-TTGGAGGGTCAACACAGTGATCGG-3'). The PCR program for *gag* and *pol* genes included one cycle of 95°C for 5 mins, 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 6 mins.

RT-PCR for detecting PERV mRNA. The PCR system contained 10×RNA PCR buffer, 10 mmol/l dNTPs, 25 mmo/l $MgCl_2$, RNase inhibitor, 2 U AMV Taq and AMV RT (TaKaRa), 15 pmol of each primer, template, and distilled H_2O to a final volume of 25 μl. Primers specific for *gag* and *pol* genes were the same as above. GAPDH (573 bp) was used as an internal standard and amplified with primers: GAPDH-forward, 5'-ATCACCATCTTCCAGGAGC GAGA-3'; GAPDH-reverse, 5'-GCTTCACCACCTTCTTGATGT CA-3'. RT-PCR profile for *gag* and *pol* genes included 50°C for 30 mins, 94°C for 2 mins, 95°C for 5 mins; 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and final extension of 72°C for 6 mins. RT-PCR products were analyzed by 2% polyacrylamide gel electrophoresis.

Immunohistochemistry. All examined tissues of nude mice were formalin-fixed and embedded in a single paraffin block. Rabbit anti-PERV-*gag* antibody (1:400) and HRP-anti-rabbit IgG (1:1000) (Santa) were used to detect PERV-*gag* protein.

Results and Discussion

The discovery of PERV infectivity has raised concerns about the biosafety of pig-to-human xenotransplantation. However, the transmission of PERV has not been observed yet in humans exposed to the porcine tissues (McKane *et al*., 2004; Denner, 2003). PERV can infect many different human primary cells and cell lines *in vitro*. In this study, we examined the possibility that PERV originated from porcine cell lines could infect human cells *in vivo*. The nude mice model was used to simulate the condition of immunodeficiency of the recipient after xenotransplantation.

Our previous study showed that PERV could infect SMMC-7721 cells *in vitro* (Zhang *et al*., 2006). To detect the infectivity of PERV to human cells *in vivo*, PK15 cells and SMMC-7721 cells were implanted synchronously in the nude mice. Tumor in the left axilla of nude mice appeared 3 weeks after the SMMC-7721 cells injection and the mass in the right axilla appeared 4 weeks later. All tumors appeared at the inoculation site 6–8 weeks after the transplantation and their size were about 2×1.5×0.5 cm and 2.5×2.5×0.8 cm in the left and right axilla, respectively.

PERV *gag* and *pol* genes were considered to be the second marker to confirm PERV infection (Specke *et al*., 2001). Because pig mitochondrion COII gene can be used as a specific marker to distinguish the microchimerism of pig cells from PERV infection, we included the analysis of pig COII gene expression. Our results showed that pig COII gene (377 bp) was present in the masses of the right and left axilla of nude mice, but not in the remaining animal tissues (Fig. 1). PERV *gag* and *pol* genes were detected in all tested tissues except the liver (Fig. 2). In addition, PERV *gag* and

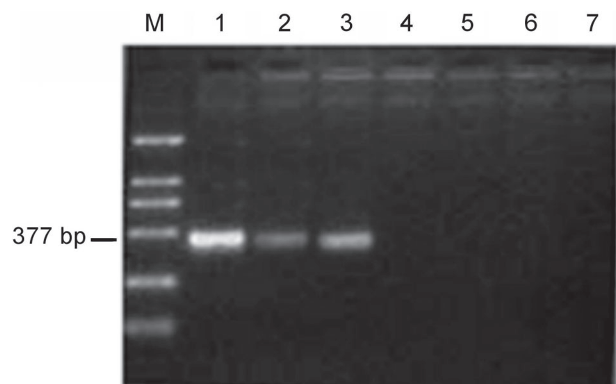

Fig. 1

Agarose gel electrophoresis of PCR products specific for pig COII gene

Size markers (lane M), PK15 cells (lane 1), axilla grafted with SMMC-7721 cells (lane 2), axilla grafted with PK15 cells (lane 3), kidneys (lane 4), lungs (lane 5), heart (lane 6), liver (lane 7).

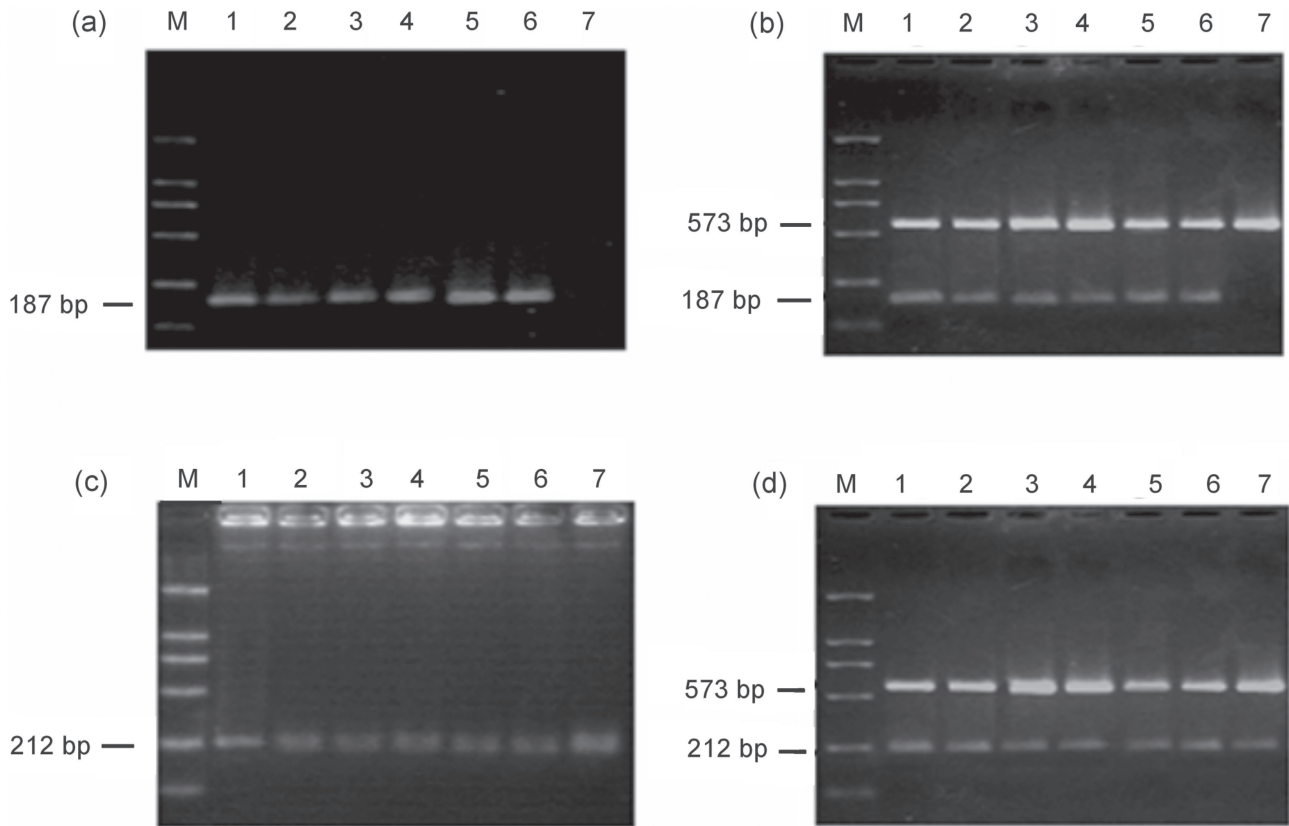

Fig. 2

Agarose gel electrophoresis of PCR products specific for PERV *gag* and *pol* genes

PERV *gag* gene (a), PERV *gag* mRNA (b), PERV *pol* gene (c), and PERV *pol* mRNA (d). Size markers (lane M), PK15 cells (lane 1), axilla grafted with SMMC-7721 cells (lane 2), axilla grafted with PK15 cells (lane 3), kidneys (lane 4), lungs (lane 5), heart (lane 6), liver (lane 7).

pol genes in the infected tissues were sequenced and identified as the corresponding regions of virus from PK15 cells (data not shown). Therefore, we concluded that all detected *gag* and *pol* genes originated from PERV rather than from the genome of murine endogenous retrovirus. According to the PCR analysis for pig COII, PERV *gag* and

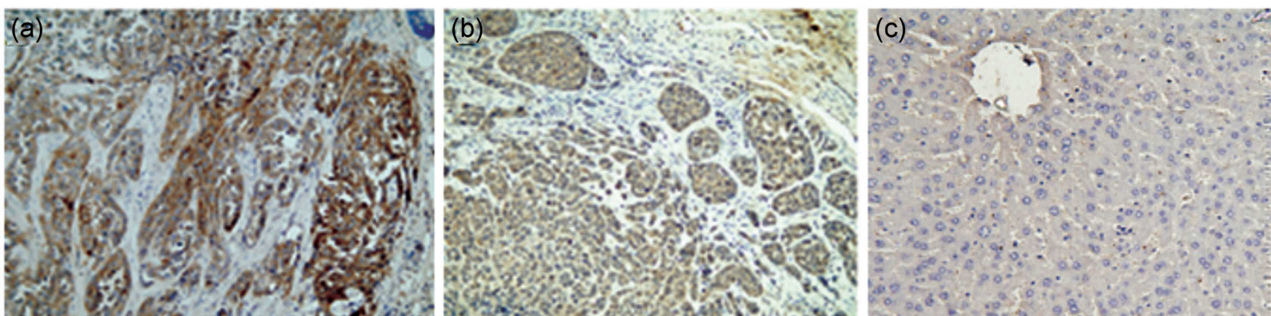

Fig. 3

Immunochemical detection of PERV *gag* protein

The axilla grafted with SMMC-7721 cells (a), PK15 cells (b), and the liver of infected mouse (c). Magnification 200x.

pol genes, PERV could be transmitted from pig cells to mouse organs and therefore additional experiments are required to clarify whether SMMC-7721 cells were infected with PERV due to the microchimerism. PERV *gag* gene was not detected in liver what implied that the PERV genome was incomplete in some infected mouse tissues and therefore PERV proteins could not be synthesized efficiently. This result can also explain the lack of PERV *gag* expression in the liver (Fig. 3c). PERV Gag was detectable in the mass formed by injection of SMMC-7721 cells indicating a possibility of PERV transmission to human cells after xenotransplantation (Fig. 3a). However, the transcription of PERV and expression of *gag* protein were observed in other mouse tissues suggesting that PERV genes in infected mouse tissues were active and efficient.

To evaluate the potential risk of PERV trans-species transmission *in vivo*, Specke *et al*. (2002) performed related experiments using the small laboratory animals and non-human primates, such as the naive and immunosuppressed rat, guinea pig, mink, rhesus monkey, pig-tailed monkey, and baboon. Their results showed that none of the small animals or non-human primates produced antibodies against PERV or integrated PERV proviral DNA in their genomic DNA for 3 months after virus inoculation (Specke *et al*., 2002). In a SCID mouse model, PERV was transmitted from pig to the mouse cells and integrated in the mouse genome, but PERV could not replicate and express the proteins (Deng and Tuck, 2000). Porcine fetal islets were transplanted under the kidney capsule of immunocompetent or nude athymic rats and the PERV expression was induced after transplantation, but it was independent on both the unspecific inflammatory response and the specific T-cell-mediated rejection process (Schmidt *et al*., 2005). Considering the results of previous studies and our experiments as well, microchimerism and PERV transmission were frequently observed in the small experimental animals like mice grafted with pig cells. The apparent difference in the outcome of the *in vivo* data from other studies might be explained by an efficient elimination of the virus by the innate or adaptive immunity of the animals and the different level of hypoxia in the xenograft. Our experiments indicate that the risk of PERV transmission in the xenotransplantation must be taken into consideration and a further study should focus on the systematic analysis of PERV infection *in vivo*.

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