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SNU 미니돼지 유래 돼지내재레트로바이러스의 시험관내 사람세포주에서의 증식부전

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No Evidence of the Productive Replication of Porcine Endogenous Retrovirus (PERV) from SNU Miniature Pigs in Human Cell Line

Background: The presence of porcine endogenous retrovirus (PERV) has been considered as one of the main hurdles to transplant pig's organs or tissues to human beings. There has been no report that PERV infection is associated with human diseases. Because pigs have their own characteristics of PERV according to pig strain, it is necessary to analyze the infectivity of PERV from SNU miniature pig to human cells for future utilization as a transplantation donor.

Materials and Methods: Human cell lines were infected with culture supernatant from porcine cell line or immunomodulator-stimulated peripheral blood mononuclear cells (PBMC) of SNU miniature pigs. They were also co-cultured with PBMC or islet cells of SNU miniature pigs. The presence of PERV genes and general pig marker gene in cells was determined by nested PCR with primer set for PERV *pol* and pig mitochondrial cytochrome oxidase II (COII), respectively.

Results: Infection test with the culture supernatant from PBMC of SNU miniature pigs showed that PERV *pol* but not COII was detected only in a few cases, but there was no uniform infection pattern in scope of stimulators and cell types. PERV *pol* was not demonstrated in co-cultures of human cell line with PBMC or islet cells from SNU miniature pigs after 80 days of co-cultures.

Conclusions: *In vitro* infectivity test suggests that PERV from SNU miniature pig might not replicate productively in human cell lines although it could infect human cells and integrate into chromosome.

Key Words: Porcine endogenous retrovirus, Infectivity, Human cell line, Co-culture, miniature pig, Xenotransplantation

Introduction

Xenotransplantation using pig organs and tissues is considered as a possible alternative to overcome the increasing needs for human transplantation

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donors (1). However, there has been a report suggesting that the infectivity of porcine endogenous retrovirus (PERV) to human cells may be connected with the potential risk of zoonosis (2), and this led to the (worldwide) prohibition of xenotransplantation with pigs due to its unique nature of being endogenously present in every porcine tissue and its ability to integrate into human genome. Although PERV-derived proteins could inhibit the function of immune cells *in vitro* (3), there has been no report on the effect of natural PERV infection on genetic or cellular functional alteration after PERV genome integration into normal human cells. It was reported that some inbred miniature pig failed to produce human-tropic replication-competent PERV (4). There is no evidence that PERV infection is associated with human diseases (5). PERV production is different from strain to strain (3, 6), and it is necessary to assess the characteristics regarding the infectivity of PERV from pigs to human cells as a possible donor source. To evaluate the long-term result of PERV infection on human cells, they were co-cultured with peripheral blood cells or islet cells from SNU miniature pigs, and were continually cultured for 3 to 6 months in this study.

Materials and methods

1. Cell culture

PK-15 (ATCC CCL-33), ECV304 (ATCC CRL-1998), HEK-293 (ATCC CRL-1573), T47D (ATCC HTB-133), HEL299 (ATCC CCL-137), and U373MG (ATCC HTB-17) cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS) at 37℃ in CO_2 incubator. Jurkat (ATCC TIB-152), Raji (ATCC CCL-86), and THP-1 (ATCC TIB-202) were cultured in RPMI1640 containing 10% FBS at 37℃ in CO_2 incubator.

2. Pig cells preparation and PERV infection

Porcine peripheral blood was obtained from a gnotobiotic SNU miniature pig breed at the Center for Animal Resource Development, Seoul National University College of Medicine. Peripheral blood mononuclear cells (PBMCs) were collected at the interface after the centrifugation on Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) at 400 xg for 30 minutes. Islet cells of SNU miniature pigs were prepared as previously described (7).

The culture supernatant of PBMC stimulated with PMA (17 ng/mL), PHA (8 ng/mL), PMA and PHA, LPS (17 ng/mL), or PGE2 (17 ng/mL) was harvested after the indicated days' culture and passed through a 0.2 μm filter (Nalgene, Rochester, NY, USA) to remove the cell debris. Each inoculum was then used to infect human cell lines with 8 μg/mL polybrene. All the reagents were purchased from Sigma (St. Louis, MO, USA). After 2 days of culture, the cells were harvested and tested for the presence of PERV by nested PCR.

For the infectivity test of pig PBMC by co-culture method, 1×10^6 PBMCs/mL was mixed with 6×10^5/mL THP-1, 8×10^5/mL Raji, and 5×10^5/mL Jurkat, respectively. The same number of PBMCs was put onto the monolayer-cultured cells, 9×10^5/mL HEK-293, 7×10^5/mL ECV304, 1×10^6/mL T47D, 9×10^5/mL HEL299 and 2×10^5/mL U373MG, respectively. After co-culture for 24 hours, the culture media was removed and replaced with the fresh medium. Then the infected cells were subcultured every 3 days and grew in the culture medium for 3 to 6 months.

For the infectivity test of pig islet cells by co-culture method, 1×10^3 IEQ was stimulated with PMA (17 ng/mL), PHA (8 ng/mL), LPS (17 ng/mL), or PGE2 (17 ng/mL), and put onto HEK-293 cultures. After co-culture for 24 hours, the culture media was removed and replaced with the fresh medium. Then the infected cells were subcultured every 3 days and grew in the culture medium for 3 to 6 months.

3. Polymerase chain reaction (PCR)

DNA of cells was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). PCR was carried out in AccuPower PCR premix (Bioneer, Seoul, Korea) containing 10 pmol of forward and reverse primers of PERV *pol* or pig COII summarized in Table 1 (8), and 10 pg of DNA in a total volume of 20 μL. PCR was performed with 30 cycles at 94℃ for 30s, at 55℃ for 30s, and at 72℃ for 30s for *pol* amplification, and with 30 cycles at 94℃ for 1min, at 64℃ for 1min, and at 72℃ for 1min for COII amplification. The products were then separated on 2% agarose gel (SeaKem, Rockland, ME, USA).

Table 1. Primer sets used for nested PCR in this study

	primary	nested
pol	5' GCA TTC AGT GCT GCT ACA AC 3'	5' GCT ACA ACC ATT AGG AAA ACT AAA AG 3'
	5' ATT GGA CAG GAA CTA GGA TG 3'	5' ACC CAG GAC TGT ATA TCT TGA TCA G 3'
COII	5' CTT ACC CTT TCC AAC TAG GCT TC 3'	5' CAC ACA CTA GCA CAA TGG ATG CC 3'
	5' TTC GAA GTA CTT TAA TGG GAC AAG 3'	5' GAG GAT ACT AAT ATT CGG ATT GTT AT 3'

COII, cytochrome oxidase II

4. Reverse transcription polymerase chain reaction (RT-PCR)

RNA was prepared from 250 μL of plasma or culture supernatant by QIAamp MinElute Media kit (Qiagen). After mixing prepared RNA with primer $p(dT)_{10}$ (Roche, Mannheim, Germany), the mixture was incubated at 70℃ for 5 min and then placed on ice. AccuPower RT premix (Bioneer, Seoul, Korea) was used for cDNA synthesis. For cDNA synthesis, one cycle was performed at 42℃ for 60 min; RTase inactivation step was carried out one cycle at 94℃ for 5 min.

Results

1. Detection of PERV *pol* and pig cytochrome oxidase II (COII)

Nested PCR for PERV *pol* and pig COII was performed with the serially diluted samples containing known amount of cellular DNA of PK-15 for determination of the detection limit. *Pol* and COII could be detected up to 0.1 pg and 1×10^{-3} pg, respectively.

2. Detection of PERV virion RNA

For the test of the presence of PERV virion, RNA was prepared from 250 μL of pig plasma or the culture supernatant of PBMCs with DNase treatment, and RT-PCR was performed for PERV *pol*. PERV virion RNA was not detected in pig plasma by RT-PCR (Fig. 1 A). It was detected in the culture supernatant from PMA- or PHA-stimulated PBMCs after 6 days' culture, but not from PMA plus PHA, LPS, or PGE2-treated ones (Fig. 1B).

3. Detection of PERV in human cell lines after infection with the culture supernatant of stimulator-activated PBMC from SNU miniature pig

Human cell lines, ECV304, HEK-293, THP-1, Raji, and Jurkat were infected with the prepared culture supernatant for the indicated days. The presence of PERV was detected by nested PCR with PERV *pol* primer set in DNA from the human cell lines after 2 days' cultures. The presence of pig material in human cells was also detected by nested PCR with pig COII primer set in the same material. Experiments were repeated three times with the different pig's PBMC. Both *pol* and COII were detected in almost all samples in experiment 1 and 3, while one of them or none were detected in some samples in day 1 cultures in experiment 2 (Table 2). There was no consensus result regarding the presence of PERV in any particular combination of the stimulator and the cells.

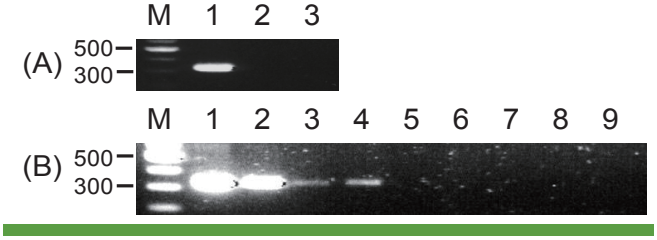

Figure 1. Detection of PERV RNA in plasma (A) and culture supernatant of PBMC (B) from SNU miniature pig by RT-PCR.
(A) M, molecular marker; 1, PK-15; 2, plasma; 3, distilled water control
(B) M, molecular marker; 1, PK-15; 2, culture supernatant of PK-15; 3-8, culture supernatant of PBMC treated with PMA, PHA, PMA plus PHA, LPS, PGE2, and media; 9, distilled water control.

4. Detection of PERV in human cell lines after co-cultured with human cell line and PBMC from SNU miniature pig

THP-1, Raji, Jurkat, T47D, ECV304, HEL299, and U373MG were co-cultured with PBMCs from SNU minipig for the indicated days.

Table 2. Detection of PERV in Human Cell Lines Infected with Culture Supernatant of Stimulator-activated PBMC from SNU Miniature Pig*

		PMA	PHA	LPS	PGE2	PMA+PHA	media
Exp-1							
Day 3	ECV304	+/+	+/+	+/+	+/-	+/+	+/+
	HEK-293	+/-	+/-	+/+	+/+	+/+	+/-
	THP-1	-/-	+/-	+/-	+/-	+/-	+/-
	Raji	+/+	+/+	+/+	+/+	+/+	+/+
	Jurkat	+/+	+/+	+/+	+/-	+/+	+/-
Day 6	THP-1	+/-	+/+	+/-	+/+	+/+	+/+
	Raji	+/+	+/+	+/+	+/+	+/+	+/+
	Jurkat	+/-	+/+	+/-	+/+	+/+	+/-
Day 9	THP-1	-/-	+/-	-/-	-/-	+/+	-/-
	Raji	-/-	-/-	-/-	-/-	+/+	+/-
	Jurkat	+/+	-/-	-/-	-/-	+/-	-/-
Exp-2							
Day 1	THP-1	-/-	-/-	-/-	-/-	-/-	-/-
	Raji	+/+	-/-	-/-	-/-	+/-	-/-
	Jurkat	+/+	-/-	-/-	-/-	-/-	-/-
	HEK-293	-/-	-/-	-/-	-/-	-/-	-/-
	ECV304	-/-	-/-	-/-	-/-	-/-	-/-
Day 7	THP-1	+/+	-/-	+/+	+/-	+/+	+/+
	Raji	-/-	-/-	+/-	-/-	+/+	-/-
	Jurkat	-/-	+/-	-/-	+/+	+/-	-/-
	HEK-293	-/-	-/-	+/-	-/-	-/-	-/-
	ECV304	+/+	+/+	+/-	+/-	+/-	+/+
Exp-3							
Day 2	hPBMC	-/-	+/-	+/-	+/-	+/+	+/-
	THP-1	+/+	+/-	+/-	+/-	+/+	+/-
	Jurkat	+/+	+/-	+/-	+/-	+/+	+/-
Day 4	THP-1	+/+	+/-	+/+	+/+	+/+	+/+
	Raji	+/+	+/-	+/+	+/+	+/+	+/+
	Jurkat	+/+	+/-	+/-	+/-	+/+	+/+
	HEK-293	+/+	+/+	+/+	+/-	+/+	+/+
Day 6	THP-1	+/+	+/-	+/+	+/-	+/-	+/-
	Raji	+/-	+/-	+/-	+/-	+/-	+/-
	Jurkat	+/+	+/-	+/-	+/-	+/+	+/-

* +/+, *pol*(+)/COII(+); +/-, *pol*(+)/COII(-); -/-, *pol*(-)/COII(-)
PMA, phorbol myristate acetate; PHA, phytohemagglutinin; LPS, lipopolysaccharide; PGE2, prostaglandin E2

Table 3. Detection of PERV in Human Cell Lines Co-cultured with Peripheral Blood Mononuclear Cells from SNU Miniature Pig*

Exp-1	Day 2	Day 30	Day 87	Day 98	Day 127	Day 147	Day 162		
Jurkat	+/+	ND	ND	ND	-/-	ND	-/-		
T47D	+/-	ND	-/-	-/-	-/-	-/-	-/-		
ECV304	+/+	-/+	-/-	-/-	-/-	-/-	-/+		
HEL299	+/+	+/+	-/-	ND	ND	-/-	-/+		
U373MG	+/-	ND	ND	-/-	-/-	-/-	-/-		
Exp-2	**D-2**	**D-7**	**D-21**	**D-38**	**D-48**	**D-63**	**D80**	**D91**	**D-105**
THP-1	+/+	ND	ND	ND	+/-	-/-	-/-	-/-	
Raji	+/+	ND	ND	-/+	ND	+/-	ND	-/-	
Jurkat	+/+	ND	ND	ND	-/-	-/-	-/-	-/-	
T47D	ND	+/+	+/+	ND	ND	ND	ND	ND	-/-
ECV304	+/+	ND	+/+	ND	-/-	-/-	-/-	-/-	

* +/+, *pol*(+)/COII(+); -/+, *pol*(-)/COII(+); -/-, *pol*(-)/COII(-); ND, not done

Table 4. Detection of PERV in HEK-293 Cells Co-cultured with Stimulator-activated Islet Cells from SNU Miniature Pig*

Exp-1	Day 5	Day 13	Day 22	Day 41	Day 62	Day 79	Day 90
Media	+/+	+/+	+/+	-/-	-/-	-/-	-/-
PMA	+/+	+/+	+/+	-/-	-/-	-/-	-/-
PHA	+/+	+/+	+/+	-/-	-/-	-/+	-/-
LPS	+/+	+/+	+/+	-/-	-/-	-/-	-/-
PGE2	+/+	+/+	+/+	-/-	-/-	+/+	-/-

Exp-2	Day 30	Day 40	Day 58	Day 69	Day 90
Media	+/+	-/+	-/-	-/-	-/-
PMA	ND	+/+	-/-	-/+	-/-
PHA	+/+	-/+	-/-	-/-	-/-
LPS	-/+	-/+	-/-	-/-	-/-
PGE2	+/+	-/+	-/-	-/-	-/-

*+/+, *pol*(+)/COII(+); -/+, *pol*(-)/COII(+); -/-, *pol*(-)/COII(-); ND, not done.
PMA, phorbol myristate acetate; PHA, phytohemagglutinin; LPS, lipopolysaccharide; PGE2, prostaglandin E2

The presence of PERV was detected by nested PCR with PERV *pol* primer set in DNA from cells in culture. Experiments were repeated twice with the different pig's PBMCs, and PERV *pol* could not be detected in any sample after 80 days of co-culture (Table 3).

5. Detection of PERV in HEK-293 after co-cultured with stimulator-activated islet cells from SNU miniature pig

HEK-293 cells were co-cultured with various stimulator-activated islet cells from SNU minipig for the indicated days. The presence of PERV was detected by nested PCR with PERV *pol* primer set in DNA from cells in culture. Experiments were repeated twice with the different pig's islet cells, and PERV *pol* could not be detected in any sample after 40 days of co-culture (Table 4).

Discussion

We performed PERV infectivity tests by transmission assay and co-culture assay, and demonstrated that PERV from SNU miniature pigs did not replicate productively in human cell lines in this study. PERVs have been reported to be spontaneously produced from established porcine kidney cell line, PK-15 (9), and could infect the human cell lines *in vitro* (2). PERV subtypes, PERV-A, -B, and -C, have been identified in the genomic DNA of pigs and porcine cell lines (10, 11). The PERV-A and -B subtypes can infect human and pig cells *in vitro*, while PERV-C subtype is not able to infect human cells but can replicate in pig cell lines (2, 11). Although human-tropic PERVs have been produced from porcine cell lines (2, 11-15), molecularly cloned PERVs have had been released from human cells with low titers and limited replication competence (16-18).

To verify the contamination with porcine cells or genes during infection procedure, PCR with primers specific for pig mitochondrion cytochrome B oxidase II (COII) gene were included in the test. It was detected in the test with culture supernatant from PBMCs, but not from PK-15. These results suggest that during the activation with stimulators and the cell

preparation, a part of PBMCs were destroyed and their genes were released into the culture media and contaminated the cells. From the results on the infectivity of PERV from culture supernatants of PBMCs, PERV *pol*, but not COII, could be detected in all kinds of the tested human cell line even though in different experiment and different experiment day (Table 2). However, we could not detect PERV *pol* in these samples when cultured further (data not shown). These results suggest that PERV from PBMCs of SNU miniature pigs could infect human cell lines and integrate its genome into their chromosomes, but not replicate successfully in them. It was not clear why the detection results of PERV were different from one experiment to other (Table 2). There could be two possible explanations. One is that the characteristics of the infective viruses present in each pig were different. The other is that the virus titers released from PBMCs were very low and the amount of integrated PERV was above the detection limit of nested PCR in some cases, but not in the other cases. Therefore we introduced a more sensitive method, co-culture assay, in the detection of PERV *pol* in human cell lines after mixing with porcine cells.

We investigated the PERV infection characteristics of miniature pigs by using in vitro co-culture assays. Two independent experiments with different pig's samples were done. PERV *pol* and COII were not detected after 80 days of co-culture of PBMCs and human cell lines, and after 40 days of co-culture of islet cells and HEK-293 cells. If we assume that porcine cells did not proliferate, that PERV did not replicate in original porcine cells or human cells, that human cells divided every day, and that PERV pol detection limit by nested PCR was 0.1 pg, it can be said that after 40 days of co-culture, no PERV *pol* could be detected. When we extrapolate the above assumption, PERV from PBMCs and islet cells of SNU miniature pigs can be presumed to be unable to replicate successfully in human cell lines.

The intracellular restriction factors such as TRIM5α and APOBEC are involved in the inhibition of retroviral replication in cells (19). It is suggested that PERV does not replicate well in nonhuman primate cells (20) and also in most human cells. Because human embryonic kidney cell line, HEK-293, does not express APOBEC3G (14), it was included as target cells for transmission assay and co-culture assay in this study. Because PERV could not be detected in HEK-293 after transmission of PERV and co-culture with porcine cells, PERVs from SNU miniature pigs seems to have been released from porcine cells with very low titer so as not to infect HEK-293 cells or have defective genome that cannot replicate in them by itself. We reported previously that a large part of PERV in PK-15 cells contained the defective genome (21).

Effect of PERV infection on the growth of HEK-293 cells was reported by Yu et al. (22). Infected and uninfected HEK-293 cells showed no significant differences in morphology and total DNA, while infected HEK-293 cells doubled a little earlier than the uninfected control, and grew poorly in serum-free medium. Although PERV could not be detected in human cells in this study, PERV from SNU miniature pigs could integrate into human chromosome (Table 2) and therefore the effect of PERV integration on human cells should be elucidated in future studes. PERV genomes consist of 5' LTR, *gag, pol, env,* and 3' LTR, but pig cells contain different copy numbers of gag and *pol* genes (23), meaning that many defective PERVs are present in them at genomic level. Further study should include the analysis on the characterization of PERV construct present in SNU miniature pig to verify the capacities of viral replication at genomic level.

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