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Unusual Permeability of Porcine Endogenous Retrovirus Subgroup A Through Membrane Filters

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ABSTRACT. In xenotransplantation from pigs to humans, a bio-artificial endocrine pancreas (Bio-AEP), in which pancreatic endocrine cells are encapsulated within a semipermeable membrane of 100 nm pore size, has been developed. We evaluated the permeability of porcine endogenous retroviruses (PERVs) through membrane filters using a pseudotype virus (*LacZ*(PERV-A)) containing a viral core derived from murine leukemia virus and an envelope (Env) from PERV subgroup A. Contrary to our expectations, *LacZ*(PERV-A) lost its infectivity by filtration through a 200 nm membrane filter. This unusual phenotype was not observed in pseudotype viruses harboring Envs from other gammaretroviruses. The infectivity of *LacZ*(PERV-A) was significantly decreased by repeated freeze/thaw treatment, indicating that *LacZ*(PERV-A) was physically labile. In addition, *LacZ*(PERV-A) may be agglutinated because copy numbers of viral RNA after filtration were significantly reduced by filtration through the 200 nm membrane. This phenotype is advantageous to develop a safe Bio-AEP blocking PERV infection.

KEY WORDS: endogenous retrovirus, islet, swine, xenotransplantation, zoonosis.

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Xenotransplantation is considered to overcome the shortage of donors in organ and tissue transplantation and pigs are one of the most suitable donor animals for human recipients [4]. Specific pathogen-free pigs are available as donors; however, all pigs have infectious agents to human cells, termed porcine endogenous retroviruses (PERVs) [1, 2, 9]. PERVs are widely distributed in the porcine genomes as proviral DNA [6] and they are classified as class I endogenous retrovirus (ERV) related to the counterpart of exogenous retrovirus, genus *gammaretrovirus*, which includes murine leukemia viruses (MLVs) and feline leukemia viruses (FeLVs) [6]. Based on the differences of receptor usage [18], PERVs are divided into 3 subgroups, termed PERV-A, -B, and -C. Both PERV-A and -B infect cell lines from various mammalian species, including humans *in vitro* [6, 20], whereas PERV-C infects only cells derived from pigs. The receptor(s) for PERV-B is unknown at present; however, two receptors for PERV-A were identified in humans and named human PERV-A receptors (HuPARs) 1 and 2 [7]. The mRNA for HuPARs is expressed in many organs and tissues, including peripheral blood mononuclear cells, placenta and testis; therefore, PERV-A has the potential to be transmitted to humans *in vivo* [7]. Generally, ERVs are not pathogenic in natural hosts; however, certain ERVs are shown to be pathogenic when they infect other species as exogenous retroviruses. For example, gibbon ape leukemia virus is considered to have originated from an ERV of Asian mice and induces leukemia in gibbons [10, 21]; therefore, for application in xenotransplantation, it is necessary to block infection by PERVs.

Porcine organs and tissues express xenoantigens that induce strong immune reactions in the recipients. To overcome immunological rejection, Ohgawara and coworkers developed a bio-artificial endocrine pancreas (Bio-AEP), in which pancreatic endocrine cells are encapsulated within a semipermeable membrane [13, 14]. They found that the immune reaction between the recipient and transplanted cells was prevented by a semipermeable membrane of 100 nm pore size. In this study, using MLV-based pseudotype viruses, we investigated the permeability of PERV-A and other gammaretroviruses through membranes of different pore sizes. We found that the infectivity of the PERV-A pseudotype virus was specifically lost by filtration through a membrane of 200 nm pore size. This observation was unexpected because the pseudotype virus was considered to be smaller than 200 nm; therefore, we further studied the mechanism of the phenomenon.

MATERIALS AND METHODS

Cell cultures: Human embryonic kidney (HEK) 293T (ATCC, CRL-11268), TELCeB6/FBPERV-A [18], TELCeB6/FBFeLV-B [11], and TELCeB6/AF-7 cells [5] were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/m*l* penicillin and 100 μg/m*l* streptomycin (Invitrogen, Carlsbad, CA, U.S.A.) at 37°C in a humidified atmosphere of 5% CO_2 in air.

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Preparation of pseudotype viruses: Pseudotype viruses designated as *LacZ*(PERV-A), *LacZ*(FeLV-B), and *LacZ*(A-MLV) were collected from culture supernatants of TELCeB6/FBPERV-A, TELCeB6/FBFeLV-B, and TELCeB6/AF-7 cells, respectively. These pseudotype viruses contain the viral core derived from MLV and *LacZ* genes with a packaging signal derived from MLV as a genome. *LacZ*(PERV-A), *LacZ*(FeLV-B), and *LacZ*(A-MLV) harbor Envs of PERV-A, FeLV subgroup B (FeLV-B), and amphotropic MLV (A-MLV), respectively.

Titration of pseudotype viruses by the LacZ assay: Basically, viral samples were filtrated through a membrane filter of 800 nm pore size (PALL, Ann Arbor, MI, U.S.A.), and then immediately subjected to titration. To examine the permeability and stability of the pseudotype viruses, viral samples were treated separately as follows. (1) Filtration through membrane disk filters: viruses were filtrated through disc filters of different pore sizes (800, 450 and 200 nm) (PALL). (2) Incubation at 42°C: each virus was incubated at 42°C for 0, 1, 2, or 3 hr. (3) Freeze/thaw treatment: each virus was frozen at –80°C and then thawed at room temperature once or twice (repeatedly).

Viral infectivities of pseudotype viruses were titrated by the LacZ assay as described previously [16]. Briefly, HEK293T cells, as target cells, were subcultured on 48-multiwell plates (Asahi Techno Glass Corp., Tokyo, Japan) at a concentration of 1.0×10^5 cells/well and incubated for 24 hr before inoculation of viruses. HEK293T culture medium was then aspirated and each diluted viral sample was inoculated into cells with 8 μg/m*l* (final concentration) polybrene (hexadimethrine bromide) (Sigma-Aldrich Corp.). Four hr after inoculation, supernatants were replaced with fresh medium, and the infected cells were incubated for an additional 2 days before X-Gal staining. Assays were performed in triplicate and repeated as three independent experiments. Virus titers were determined by counting LacZ-positive blue foci and expressed as focus-forming units (f.f.u.)/m*l*.

Quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR): The viral copy number present in each filtrated sample was quantitated by real-time RT-PCR targeted for viral *LacZ* (GenBank; accession no.: NC_011601.1, protein ID: YP_002327878) gene. Quantitative real-time RT-PCR was performed using the One Step SYBR PrimeScript RT-PCR Kit (Perfect Real Time) (TaKaRa, Otsu, Japan) and Retrovirus Titer Set (for real-time PCR) (TaKaRa) according to the manufacturer's instructions. PCR and the resulting increase in reporter fluorescent dye (SYBR Green I) emission were monitored in real time using a 7000 Real Time PCR system (Applied Biosystems, Foster City, CA, U.S.A.). The primer pair (forward primer positioned at 1541 to 1560; 5'-CTGTGCCGAAATGGTCCATC -3', and reverse primer positioned at 1591 to 1572; 5'- GCGTCTCTCCAGGTAGCGAA -3') was designed using the Primer Express Program (Applied Biosystems). The thermal cycling condition consists of 1 cycle at 42°C for 5 min, 1 cycle at 95°C for 10 sec, and 40 cycles at 95°C for 5 sec and 60°C for 31 sec. The standard curve was generated by serial dilution of a plasmid containing *LacZ* cDNA to quantify viral *LacZ* mRNA. All values indicate copy numbers of viral *LacZ* mRNA contained in one microliter of supernatant. Quantitation was performed for three independent experiments, and all values are presented as the means ± SEM.

Electronic microscopy (EM): The morphology and size of *LacZ*(PERV-A) and *LacZ*(FeLV-B) were compared by EM. TELCeB6/FBPERV-A and TELCeB6/FBFeLV-B cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in the same buffer, dehydrated, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate, and observed with an electron microscope.

Statistical analysis: Differences in viral titers were assessed by one-way ANOVA, followed by the Tukey-Kramer multiple comparison test. All differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

PERV-A is an ERV related to exogenous gammaretroviruses and the size of the virion is approximately 90 to 120 nm in diameter [8]. To examine whether PERV-A could flow through a membrane filter of 200 nm pore size, we prepared a pseudotype virus, termed *LacZ*(PERV-A), harboring PERV-A Env. Contrary to our expectations, *LacZ*(PERV-A) was significantly attenuated (over 2.0×10^2-fold) and lost its infectivity by filtration through a membrane filter of 200 nm pore size ($P<0.05$) when compared with the infectivity of the virus after filtration through a membrane filter of 800 nm pore size (Fig. 1). We used membrane disc filters of different pore sizes (800, 450 and 200 nm) from the same manufacturer and the material of all filters was hydrophilic polyether sulfone; therefore, it was considered that this difference was not due to nonspecific adsorption of the virus to the 200 nm filter. This unusual phenotype was PERV-A Env specific, because the infectivities of *LacZ*(FeLV-B) and *LacZ*(A-MLV) were not affected by filtration through the 200 nm membrane filter ($P>0.05$) (Fig. 1) and only Env differed in respective pseudotype viruses. To support our observations, the stability and size exclusion of *LacZ*(MLV-A) have been reported by Pizzato *et al.* [15]. Their results are consistent with our observations; infectivity of *LacZ*(A-MLV) did not fall after filtration through a 200 nm filter when compared with the titer after filtration through 450 nm [15].

Particles of gammaretrovirus (including infectious class I ERVs) consist of a viral core and Env. Env consists of two subunits; surface (SU) and transmembrane (TM) units of glycoproteins. SU and TM subunits interact with a disulfide bond between the SU CX_2C-motif and TM CX_7C-motif [19]. The principal mechanism of gammaretrovirus infection is conserved and the processes are as follows [19]: (1) viruses bind to host cells by ligation of SU Env to a specific receptor expressed on the cells, (2) the conformation of the SU Env changes, and calcium ions flow into Env, activating

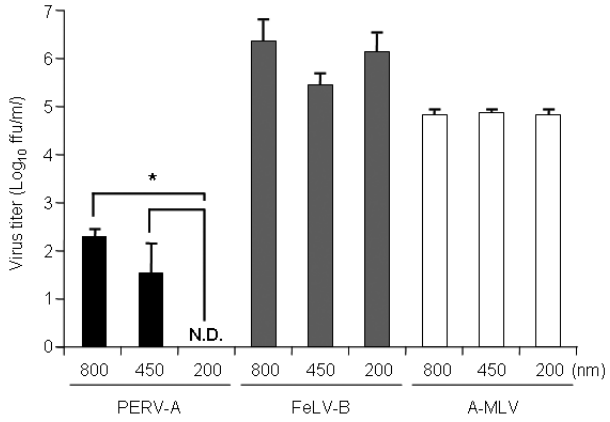

Fig. 1. Titers of pseudotype viruses filtrated thorough membrane disc filters of different pore sizes (800, 450 and 200 nm). Viral titers were determined by the LacZ assay. Black, grey and white bars indicate *LacZ*(PERV-A), *LacZ*(FeLV-B), and *LacZ*(A-MLV), respectively. Titers expressed as f.f.u./m*l* are shown as the means ± SEM based on three independent triplicate experiments. Asterisks indicate significant differences (P<0.05).

isomerase enzymes, leading to a break in the interaction between SU and TM, (3) TM is exposed outside of SU and attaches to the host cell membrane; (4) finally, viral and cellular membranes fuse with each other, enabling the viral core to enter the host cells. In filtration using membrane disc filters, the viral solution in the injection cylinder is subjected to pressure. The degree of pressure should increase as the pore size of the filter decreases. External physical forces, including high pressure, may induce the conformational change of SU without ligation to a specific receptor, leading to the separation of SU and TM Env proteins. Therefore, we hypothesized that PERV-A Env is more labile against physical forces than other gammaretroviruses, and compared the stability of pseudotype viruses against different external physical forces; incubation at 42°C and freeze/thaw treatment. As a result, the infectivity of all three pseudotype viruses diminished as incubation at 42°C proceeded (Fig. 2A). When comparing the titers of 0-hr incubation with those of 3-hr incubation, the titers significantly reduced 9.0×10^2-, 7.1×10^3-, and 6.8×10^3-fold in *LacZ*(PERV-A), *LacZ*(FeLV-B), *LacZ*(A-MLV), respectively (P<0.05). With two freeze/thaw treatments, the infectivities of *LacZ*(FeLV-B) and *LacZ*(PERV-A) markedly decreased; however, the reduction of titers was only significant in *LacZ*(PERV-A) (P<0.05) (Fig. 2B). To support our results, the stability of an A-MLV pseudotype virus after incubation at 37°C and freeze/thaw treatment have been reported by Strang *et al.* [17]. In their report, the infectivity of A-MLV pseudotype virus diminished after incubation at 37°C for 6 hr, whereas it was stable with two freeze/thaw treatments [17]. From the freeze/thaw treatment data obtained in this study, the loss of infectivity of *LacZ*(PERV-A) by filtration can be explained in part by the physical instability of *LacZ*(PERV-A).

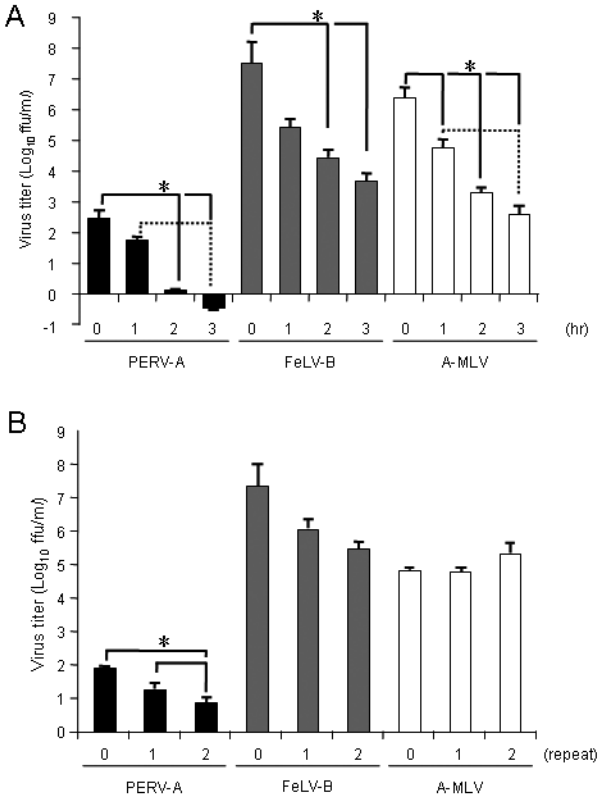

Fig. 2. (A) Titers of pseudotype viruses after incubation of the viruses at 42°C. (B) Titers of pseudotype viruses after freeze/thaw treatment. Black, grey and white bars indicate *LacZ*(PERV-A), *LacZ*(FeLV-B), and *LacZ*(A-MLV), respectively. Viral titers were determined by the LacZ assay. Titers expressed as f.f.u./m*l* are shown as the means ± SEM based on three independent triplicate experiments. Asterisks indicate significant differences (P<0.05).

To explore another possible reason for the phenomenon, we quantified copy numbers of pseudotype viruses after filtration. The mRNA encoding the *LacZ* gene in pseudotype viruses was measured using quantitative real-time RT-PCR (Fig. 3). It was considered that gammaretroviral Gag and Pol proteins are produced and processed in the TELCeB6 cells, regardless of the type of retroviral Env. The processed viral Gag-Pol proteins formed viral cores which are released from the TELCeB6 cells without viral Env proteins; therefore, it was expected that the numbers of viral particles (including defective viral particles) were similar in *LacZ*(PERV-A), *LacZ*(FeLV-B) and *LacZ*(A-MLV) released from the producer cells. Actually, viral copy numbers of *LacZ*(PERV-A) were comparable to those of other pseudotype viruses (Fig. 3). However, the amount of *LacZ*(PERV-A) was significantly decreased 7.6-fold (87% reduction) (P<0.05) after filtration through the 200 nm filter when comparing an 800 nm filter with a 200 nm filter, whereas the copy numbers of *LacZ*(FeLV-B) and *LacZ*(A-MLV) were not significantly changed (P>0.05) (Fig. 3).

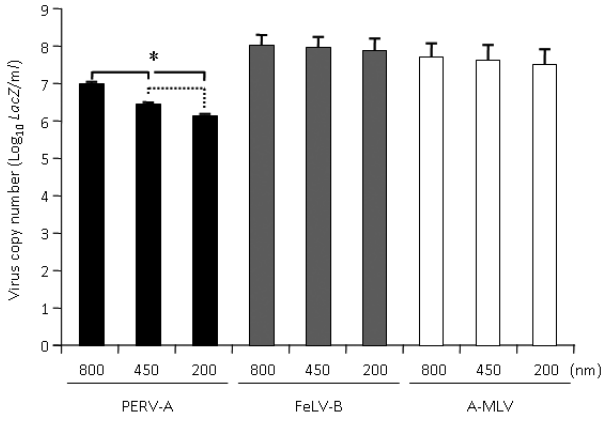

Fig. 3. Copy numbers of pseudotype viruses filtrated thorough membrane disc filters of different pore sizes (800, 450 and 200 nm). Copy numbers of *lacZ* mRNA in pseudotype viruses were quantitated by real-time RT-PCR. Black, grey and white bars indicate *LacZ*(PERV-A), *LacZ*(FeLV-B), and *LacZ*(A-MLV), respectively. Copy numbers of pseudotype viruses expressed as $\log_{10}$ (*LacZ*/m*l*) are shown as the means ± SEM based on three independent experiments. Asterisks indicate significant differences (P<0.05).

These data indicate that at least 87 % *LacZ*(PERV-A) particles were trapped on the 200 nm filter. In Table 1, we also calculated the ratios of viral titers versus viral copy numbers based on the results of Figs. 1 and 3. The titer/copy number ratios of *LacZ*(FeLV-B) and *LacZ*(A-MLV) are similar and basically constant regardless of the membrane pore sizes. In contrast, the titer/copy number ratio of *LacZ*(PERV-A) was lower than those of other pseudotype viruses and decreased after filtration through a 200 nm filter. These data indicate that the proportion of infectious *LacZ*(PERV-A) particles is reduced by filtration through a 200 nm filter.

Finally, we confirmed the morphology and size of pseudotype viruses in the culture by EM. There was no morphological difference between *LacZ*(PERV-A) and *LacZ*(FeLV-B), and both viruses were 80–100 nm in diameter (Fig. 4).

The precise mechanism of the loss of *LacZ*(PERV-A) infectivity by filtration through a membrane disc filter of 200 nm pore size is unclear at present; however, we suggest 2 possible reasons as follows. Firstly, *LacZ*(PERV-A) is labile against physical forces; therefore, *LacZ*(PERV-A) loses the SU from viral particles by filtration. Our freeze/thaw treatment data support the physical weakness of PERV-A Env. Secondly, from the results of real-time RT PCR, *LacZ*(PERV-A) particles may be agglutinated. One possible explanation for this observation is that viral particles were agglutinated by host factors, such as tetherin [12], which is involved in tethering viral particles at the cell surface or linking particles to prevent the spread of viral infection from infected cells to null cells [18]. *LacZ*(PERV-A) possibly incorporated certain host proteins to bind viral particles with each other to form clumps over 200 nm in diam-

Table 1. Relative infectivity of pseudotype viruses filtrated through three different pore sizes

		[a)]LacZ titer/viral copy number
LacZ (PERV-A)	–800 nm	0.32
	–450 nm	0.23
	–200 nm	0
LacZ (FeLV-B)	–800 nm	0.78
	–450 nm	0.67
	–200 nm	0.76
LacZ (A-MLV)	–800 nm	0.61
	–450 nm	0.62
	–200 nm	0.63

a) Ratios of $\log_{10}$ (LacZ virus titers)/$\log_{10}$ (genome copy numbers) are calculated based on the results of Figs. 1 and 3.

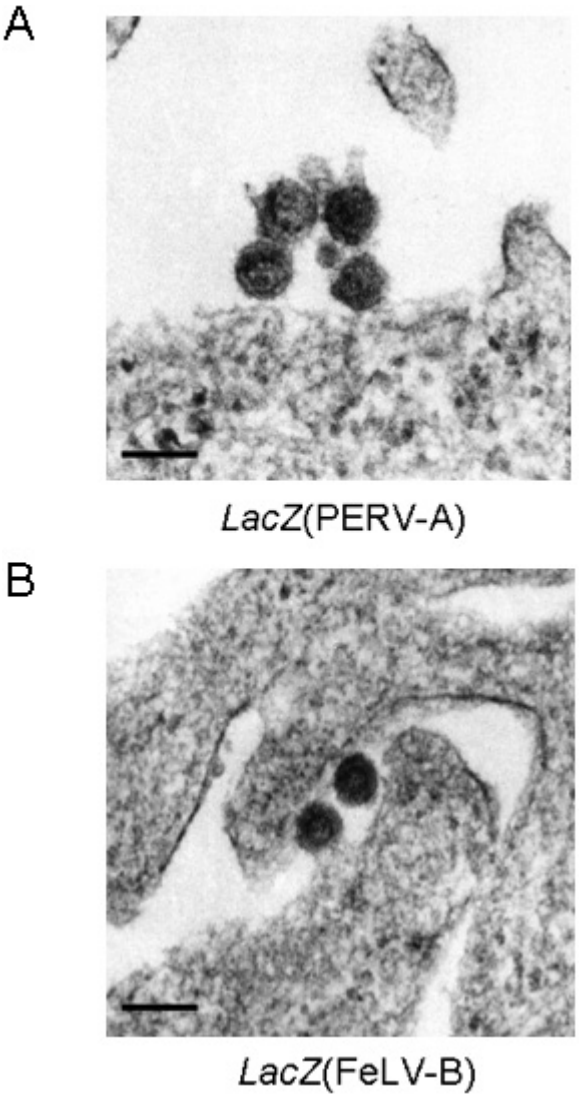

Fig. 4. Morphology and size of *LacZ*(PERV-A) (A) and *LacZ*(FeLV-B) (B) examined by EM. Scale bar is 100 nm.

eter.

In this study, we found that *LacZ*(PERV-A) lost its infectivity by filtration through a membrane filter of 200 nm pore size. We can not exclude the possibility that this phenotype is specific to the pseudotype virus produced from the producer cells (TELCeB6 cells). Unfortunately, we do not have any evidence that PERVs produced from pig pancreatic cells are the same phenotype. In addition, there might be a difference between forced filtration through the membrane filters used in this study and viral transmission through the semi-permiable membrane used for BIO-AEP. Nonetheless, this phenotype observed in this study is advantageous to develop a safe Bio-AEP which will overcome the issue of PERV infection in recipients.

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