J. Microbiol. Biotechnol. (2014), 24(4), 577–583
http://dx.doi.org/10.4014/jmb.1312.12079

jmb

Comparison of the Effects of Retroviral Restriction Factors Involved in Resistance to Porcine Endogenous Retrovirus

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Received: December 31, 2013
Revised: January 22, 2014
Accepted: January 22, 2014

First published online
January 22, 2014

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pISSN 1017-7825, eISSN 1738-8872

Three major classes of retroviral restriction factors (APOBEC3G, Tetherin, and TRIM5α) have been identified in mammals. Restriction factors are cellular proteins that are able to limit viral replication by targeting specific steps of the viral life cycle. To evaluate which restriction factor is the most effective to inhibit the replication of porcine endogenous retroviruses (PERVs), the antiviral activity of each restriction factor was compared. In pseudotype assay, the antiviral activity of human tetherin against PERV pseudotype was slightly weaker than that of human APOBEC3G (hA3G). A combination of tetherin and hA3G was more potent than each individual restriction factor. We questioned whether a combination of tetherin and hA3G could also inhibit the spreading replication of PERV. In agreement with the pseudotype assay, two restriction factors inhibit infectious PERV replication in a spreading infection. In this study, hA3G could strongly inhibit the replication of PERV, but tetherin modestly restricted it. Based on these results, we concluded that a combination of tetherin and hA3G is the most effective way to restrict PERV. A combination of different restriction factors will encourage the development of a new approach to treat retroviral disease.

Keywords: APOBEC3G, PERVs, tetherin, xenotransplantation

Introduction

Porcine endogenous retroviruses (PERVs) present a unique concern associated with xenotransplantation, because they have been shown to infect certain human cells *in vitro* [1, 15, 19]. PERVs are released from porcine cell lines, including pig kidney cell lines PK15 and MPK, and mitogenically activated peripheral blood mononuclear cells (PBMC). PERVs are classified as endogenous viruses of the family Retroviridae and genus *Gammaretrovirus*; additionally, they are closely related to mammalian type-C retroviruses, such as the gibbon-ape leukemia virus, and the murine leukemia virus. Two classes of infectious human-tropic replication-competent (HTRC) PERVs (polytropic PERV-A and PERV-B), and one class of ecotropic PERV-C have been identified; the potential for recombination between ecotropic PERV-C and human-tropic PERV-A and PERV-B adds an additional level of infection risk [25]. PERVs are integrated into the genome of all pigs; therefore, the removal of all PERV-related sequences, by knockout technology or selective breeding, is impossible. Several strategies have been proposed, and could potentially be employed to reduce PERV transmission, such as transgenic pigs expressing siRNAs, the use of restriction factor human APOBEC3G, reverse transcriptase inhibitors, viral vaccines, and single domain antibodies directed against PERV Gag [3, 6, 20].

During evolution, the host has developed a variety of "restriction factors" to fight retroviral infections. Restriction factors are components of the innate immune system, and act at diverse steps in retroviral replication. Tetherin (also referred to as BST-2 or CD317) is an antiviral cellular restriction factor that blocks the release of particles of many enveloped viruses, including retroviruses (HIV-1, HIV-2, SIV, MLV, and XMRV), filoviruses (Ebola virus and Marburg virus), arenavirus (Lassa virus), and herpesvirus; it was first identified as a cellular restriction factor that blocks the release of HIV [11, 13, 21, 26, 29]. Tetherin is a 30–36 kDa type II transmembrane protein, and expression

is induced by type I IFN [14]. The sequence homologies of tetherin among different primate species are relatively low. Tetherin consists of four domains; an N-terminal cytoplasmic tail (CT), a single transmembrane domain (TM), an extracellular coiled-coil domain, and a C-terminal glycosyl phosphatidylinositol (GPI) anchor. The extracellular domain of tetherin forms stable cysteine-linked homodimers, and is modified by N-linked glycosylation. The TM and GPI anchor of tetherin were reported to be essential for antiviral activity, whereas N-linked glycosylation was dispensable. Dimerization of tetherin was shown to be essential for inhibition of HIV-1, but not for Lassa and Marburg viruses. Tetherin protein associated with lipid rafts are found at the plasma membrane, and at the trans-Golgi network (TGN). Tetherin appears to inhibit virus release, by connecting both viral and host cell membranes.

Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) consists of seven members (hA3A, hA3B, hA3C, hA3DE, hA3F, hA3G, and hA3H) in humans. Human APOBEC3G (hA3G) inhibits the infectivity of HIV-1 variants lacking a *vif* gene. hA3G is incorporated into HIV-1 particles, and then induces hypermutation of HIV-1 proviruses by editing C to U in the proviral minus strand during reverse transcription, leading to G to A hypermutation [8, 9]. These hypermutations destabilize and block the functional HIV-1 proviruses. Vif (virion infectivity factor) binds to hA3G in the infected cell, and induces its degradation by ubiquitination [7]. Wild-type HIV-1 expresses vif, which prevents packaging of hA3G into particles. hA3G is capable of inhibiting the replication of a wide variety of retroviruses (HIV, SIV, HTLV, MLV, PERV, XMRV), non-LTR retrotransposons, and LTR retrotransposons. In addition to hA3G, hA3F can also be incorporated into HIV-1 virions, and deaminate minus-strand cDNA [2, 4, 10, 12, 23, 24]. However, hA3F has been reported to be less inhibitory and less sensitive to Vif than hA3G [17, 28].

It has been reported that PERV is insensitive to restriction by divergent mammalian TRIM5α, but human and porcine tetherin can inhibit PERV release from producer cells. Recent data indicate that hA3G and poA3F can inhibit PERV replication. Although tetherin and APOBEC3 are known to be inhibitors of PERV replication, they differ in antiviral potency. In this study, to determine whether the combination of two restriction factors can give synergistic effect to inhibit PERV infectivity, tetherin, APOBEC3, and tetherin combined with APOBEC3 were each used. We showed that a combination of tetherin and APOBEC3 was more efficient than each individual restriction factor.

Materials and Methods

Cell Lines

The 293 human embryonic kidney (ATCC 1573), 293T human embryonic kidney (ATCC CRL-11268), 22Rv1 (ATCC CRL-2505; human cells chronically infected with XMRV), 293-PERV-PK-CIRCE (ECACC 97051411; human 293 cells infected with PK15-derived PERVs), and TE671 (ATCC CRL) cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cloning of the APOBEC3G and Tetherin

To amplify human APOBEC3G sequences, total RNA was extracted from the TE671 cells, using TRI-Reagent (Molecular Research Center; Cincinnati, OH, USA), according to the manufacturer's instructions. Two primer pairs were designed to amplify human APOBEC3G (GenBank Accession No. NM_021822). hA3G-HA-for 5′-GGTACCATGAAGCCTCA-CTTC-3′ (*Kpn*I restriction site is underlined), and hA3G-HA-rev 5′-CTCGAGTTGTTTTC-CTGATTC-3′ (*Xho*I restriction site is underlined) were used for RT-PCR. The PCR products of the human APOBEC3G were ligated into the pGEM-T Easy Vector System (Promega; Madison, USA). The human APOBEC3G-ligated vectors were digested with the restriction enzymes *Kpn*I and *Xho*I. C-Terminal HA-tagged human APOBEC3G was produced by introducing the *Kpn*I-*Xho*I fragment into the pcDNA3-mCAT-3×HA vector (pcDNA3-hA3G-3×HA). Similarly, the human tetherin sequence was amplified, using cDNA generated from 293. HuTHN-HA-for 5′-GGTACCATG GCATCTACTTCGTATGAC-3′ (*Kpn*I restriction site is underlined), and HuTHN-HA-rev 5′-CTCGAGTTCTGCAGCAGAGCGCTG AG-3′ (*Xho*I restriction site is underlined) were used for RT-PCR. C-Terminal HA-tagged human tetherin was produced by introducing the *Kpn*I-*Xho*I fragment into pcDNA3-hA3G-3×HA (pcDNA3-hTHN-3×HA). Stable hA3G-expressing 293 cell lines were selected using medium containing G418 (1 mg/ml), and hA3G-expressing clones were identified by western blotting using anti-HA antibody.

Western Blot Assay

Cell lysates from the pcDNA3-huTHN-3×HA and the pcDNA3-hA3G-3×HA transfected 293 cells were prepared 2 days after transfection by lysing the cells in 300 μl of mammalian protein extraction reagent lysis buffer. All samples were denatured at 100°C for 10 min, and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes and then probed with anti-HA antibodies (1:200) (Bethyl, Montgomery, USA) for 1 h or overnight, followed by a 1:5,000 dilution of goat anti-rabbit-conjugated horseradish peroxidase (Komabio, Seoul, Korea) for 1 h. The blots were visualized using 3,3-diaminobenzidine.

Virus Production and Pseudotype Assay

pCMV-VSV-G, pVPack-PERV-GP, and pCLMFG-*lac*Z (Imgenex Co.; San Diego, USA) were used to construct PERV pseudotype viruses. For the construction of XMRV and MLV pseudotypes, pCMV-VSV-G, pVPack-XMRV-GP, pVPack-MLV-GP, and pCLMFG-*lac*Z were used. To generate the PERV-*gag-pol* expressing plasmid pVPack-PERV-GP, *Not*I-*Hpa*I fragments of the amplified PCR products and the *Hpa*I-*Hpa*I fragment from the PERV full-length molecular clone were cloned into pVPack-Eco (Stratagene; La Jolla, USA). To investigate the effects of restriction factors, 293T cells were transfected with pCMV-VSV-G (1 μg), pVPack-PERV-GP (1 μg), or pCLMFG-*lac*Z (1 μg), and each restriction factor expression plasmid (0.5 μg), using the QIAGEN (Valencia, USA) PolyFect Transfection Kit. Viral supernatants were collected from each transfection culture and used to infect 293 cells that had been plated in 6-well culture dishes, at a density of 1.5×10^5 per well. The cells were infected with 1 ml of virus in the presence of 8 μg/ml polybrene for 3 h, and 2 ml of fresh medium was then added to each well. Two days after infection, the cells were fixed with 0.5% glutaraldehyde and stained to reveal the presence of β-gal activity. Infectious titers were expressed as the blue CFU count per milliliter of virus supernatant.

Q-PCR Assay

The supernatant from restriction factor-transfected 293-PERV-PK-CIRCE was titrated on 293 cells by real-time RT-PCR. The primers PERV-*gag*-for (5′-TGATCTAGTGAGAGAGGCAGAG-3′), PERV-*gag*-rev (5′-CGCACACT GGTCCTTGT-CG-3′), beta-actin-for (5′-ATCATGTTTGAGACCTTCAA-3′), and beta-actin-rev (5′-AGATGGGCACAGTGTGGGT-3′) were used. PERV copy numbers were normalized to those of beta-actin. Real-time RT-PCR was conducted, using a SYBR Premix Ex Taq II Kit (Takara; Shiga, Japan). A Thermal Cycler Dice Real Time System (Takara) was used for the thermal cycling and to record fluorescence changes.

Results

Cloning of hA3G and Tetherin

Molecular cloning of hA3G was performed by RT-PCR, using RNA extracted from TE671 cells. The coding sequence of hA3G has two amino acid substitutions, compared with the hA3G reference sequence NM_021822. It has been reported that IFNα treatment induces the transcription of tetherin. In the treatment of 293 cells with IFNα, the human tetherin mRNA increased more than 20-fold. We treated 293 cells for 24 h with 1,000 units/ml of IFNα for the cloning of tetherin, and cDNAs encoding tetherin were amplified from 293 cells. The coding sequence of human tetherin is identical to that represented by GenBank Accession No. NM_004335. The C-terminal HA-tagged hA3G and tetherin were produced by introducing

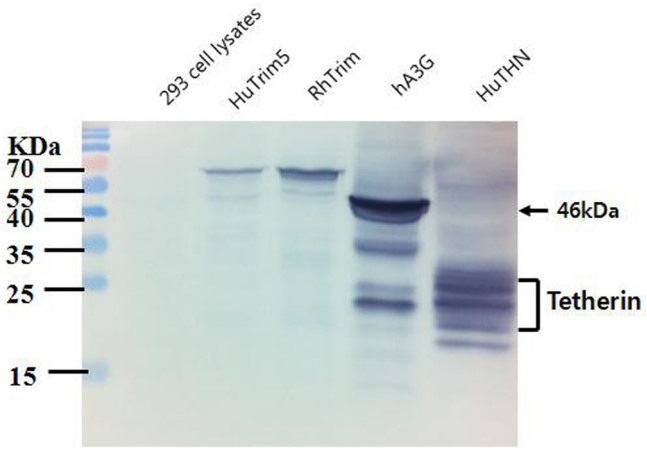


Fig. 1. Expression of hA3G and tetherin in 293 cells.
Western blot analysis of hA3G and tetherin expressed in the cells 48 h post transfection with respective plasmids. Mock-transfected 293 cells and 293 cells transiently transfected with TRIM5 were included for reference. HuTHN = human tetherin.

*Kpn*I-*Xho*I fragments into pcDNA3-mCAT-3×HA vectors. When the same amount of expression vector was transfected, hA3G and tetherin proteins were expressed at equivalent levels (Fig. 1). We examined the packaging of hA3G into PERV virions. The 293T cells were transfected with pCMV-VSV-G, pVPack-PERV-GP, or pCLMFG-*lac*Z, and various amounts of hA3G expression plasmids, using the QIAGEN PolyFect Transfection Kit. The 293T cells and virus-containing

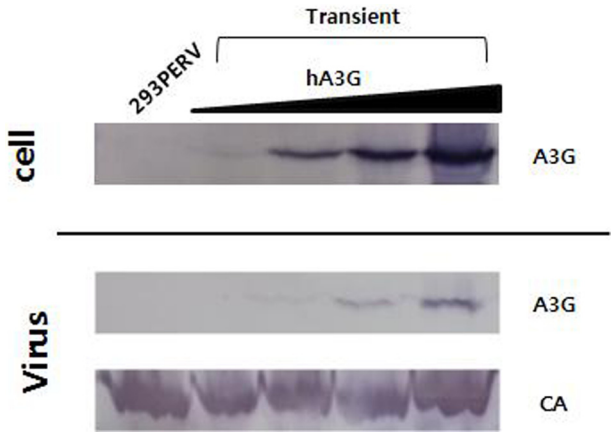


Fig. 2. Transiently expressed hA3G are packaged into PERV.
The 293T cells were transfected with increasing amounts of hA3G (0.03–1 μg). Transfected cells and virus-containing supernatants were harvested, following transfection. HA monoclonal antibody and polyclonal antibody to PERV capsid (CA) protein were used for the detection of hA3G and PERV-CA, by western blotting.

supernatants were harvested. Anti-CA and anti-HA antibodies were used for immunoblotting. As expected, hA3G was packaged into pseudotype PERV dose-dependently (Fig. 2).

Comparison of Antiviral Activity of hA3G and Tetherin

VSV-pseudotyped PERV coding for *LacZ* was produced by transfection of 293T cells with hA3G and tetherin plasmids. Pseudotype virus titers were assessed by infection of 293 cells. The antiviral activities of human tetherin against the PERV pseudotype were slightly weaker than those of hA3G. A combination of tetherin and hA3G was more potent than each individual restriction factor (Fig. 3). As shown in Figs. 3A and 3B, the infectivity of XMRV and MLV pseudotypes was similar to those seen with PERV pseudotypes.

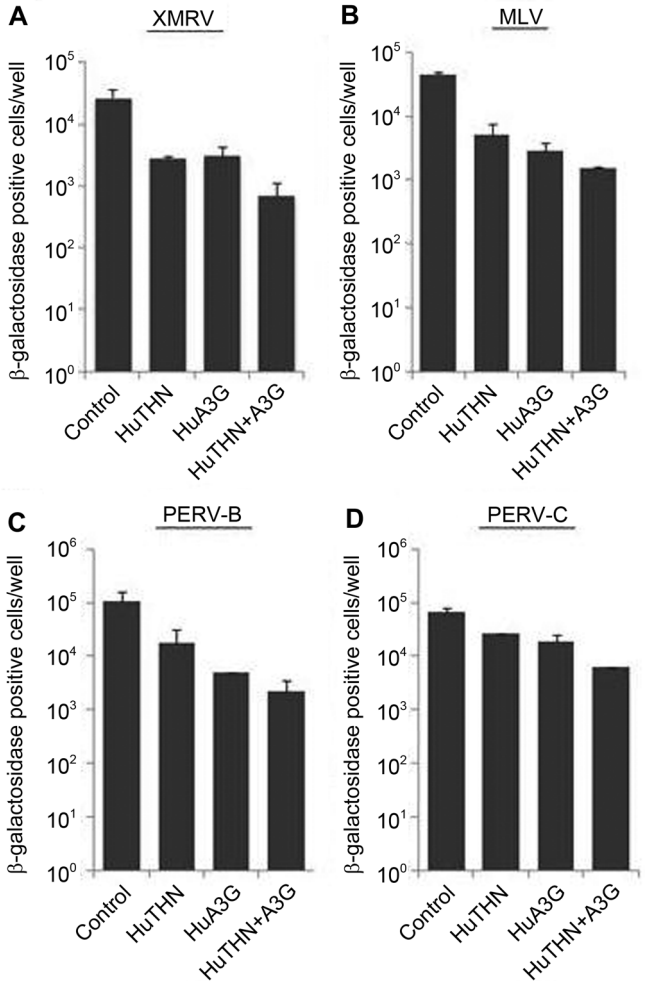


Fig. 3. The infectivity of the pseudotype viruses, produced in the presence of hA3G (0.5 μg) and human tetherin (0.5 μg), on 293 cells.

XMRV (**A**), MLV (**B**), PERV-B (**C**), and PERV-C (**D**) pseudotype viruses were harvested 48 h post-transfection, and used to infect 293 cells. After 48 h, the number of cells expressing β-galactosidase was evaluated by X-gal staining. The infectivity of the virus produced in the absence of hA3G and human tetherin was defined as the positive control. The bar represents the means of results of experiments performed at least three times.

Antiviral Activity of hA3G and Tetherin Against Replication Competent PERV

To examine whether hA3G and tetherin have inhibitory activity against infectious PERV, the expression plasmids for hA3G and tetherin were transfected into 293-PERV-PK-CIRCE cells. The supernatants from hA3G and tetherin transfected 293-PERV-PK-CIRCE cells were titrated on 293 cells. Total RNA was isolated from infected 293 cells, and the amount of PERV mRNA was demonstrated by real-time RT-PCR. Although human tetherin was efficient in reducing PERV, hA3G strongly inhibited PERV production (Fig. 4).

HA3G Expression Levels Correlate with Virus Infectivity

The hA3G stable cell lines were constructed by transfecting 293 cells with pcDNA3-hA3G-3×HA. The stable expression of hA3G was compared with the hA3G expression in 293 cells, transiently transfected with increasing

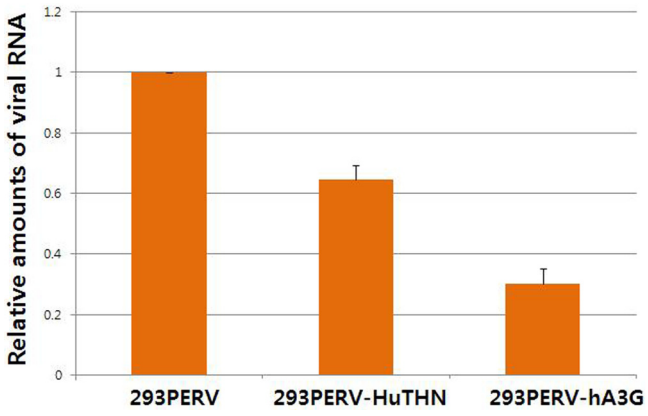


Fig. 4. The antiviral activity of hA3G is more potent than that of human tetherin.

The supernatants from hA3G and human tetherin transfected 293-PERV-PK-CIRCE were titrated on 293 cells. Viral RNA extracted from infected 293 cells was processed in a Real-time RT-PCR, based on the PERV *gag* gene. PERV copy numbers were normalized to those of beta-actin. 293PERV: 293-PERV-PK-CIRCE (ECACC 97051411; human 293 cells infected with PK15-derived PERVs). 293PERV-Te: human tetherin transfected 293-PERV-PK-CIRCE. 293PERV-APO: hA3G transfected 293-PERV-PK-CIRCE.

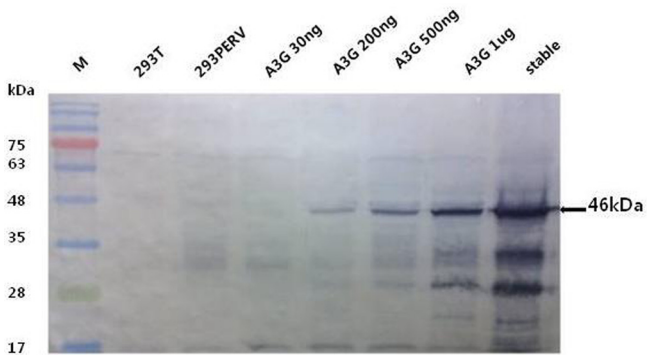


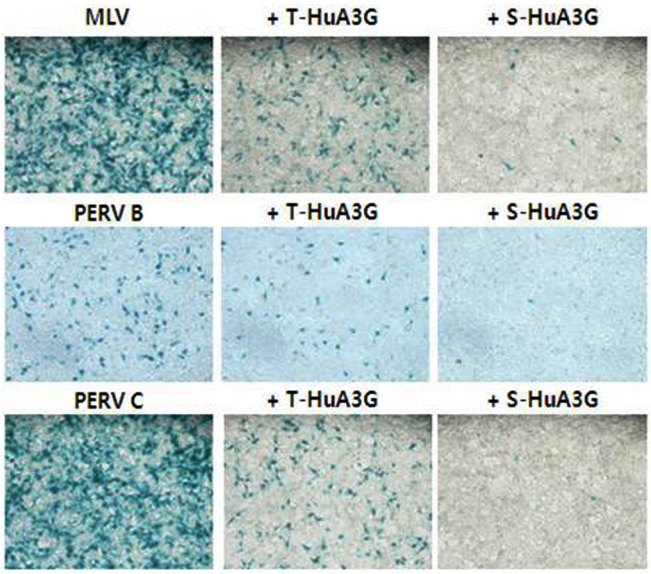
Fig. 5. Comparison of hA3G expression levels in stable cell lines with that of transiently hA3G-expressing cell lines.
Increasing levels of hA3G-expressing vectors were transfected into 293T cells, and hA3G levels were determined by western blotting.

amount of pcDNA3-hA3G-3×HA. The results show that the expression level of hA3G in stable cells was higher than that in the transiently transfected 293T cells (Fig. 5). To investigate whether the expression level of hA3G correlated with virus infectivity, a pseudotype assay was used. The infectivity of MLV and PERV pseudotypes produced from stable hA3G-expressing cell lines was much lower than the pseudotype from transiently transfected cells (Fig. 6).

Discussion

During mammalian evolution, a variety of mechanisms have arisen to limit retroviral replication. Retrovirus replication can be restricted by cellular factors, such as APOBEC3G, Fv1, TRIM5α, and tetherin [8, 16, 18, 26]. It appears that PERVs are insensitive to restriction by TRIM5α, but human APOBEC3G strongly restricts PERVs [5, 27]. We tried to reduce PERV zoonoses during xenotransplantation, by expressing different combinations of restriction factors in virus-producing human cells, to develop a novel strategy to prevent PERV transmission.

Human APOBEC3G and tetherin sequences were amplified from suitable total RNA extracts by RT-PCR. Expression of hA3G and tetherin in cell lysates was determined by western blot assay (Fig. 1). Tetherin signals were diffuse, due to heterogeneity in glycosylation, with major signals in the range of 20–34 kDa. hA3G was also expressed and packaged into the PERV pseudotype. The amounts of hA3G in the PERV pseudotype were directly proportional to the intracellular protein levels (Fig. 2). Here, we compared the antiretroviral activity of hA3G and human tetherin. We found that both hA3G and tetherin inhibited PERV infection, as expected, but hA3G was a significantly more potent inhibitor than human tetherin, in a single cycle



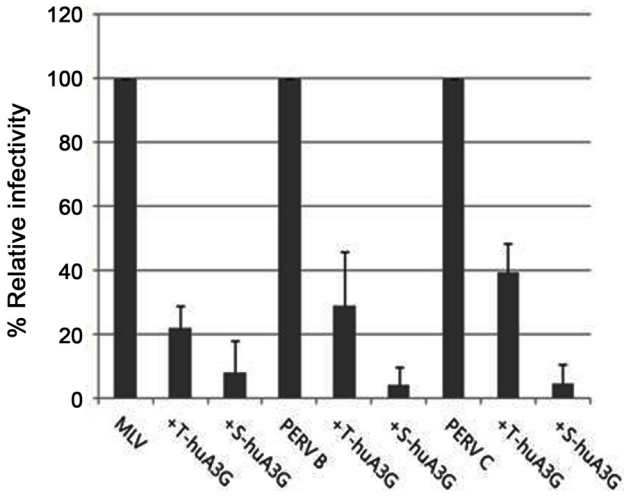


Fig. 6. hA3G expression levels correlate with virus infectivity.
The infectivity of the pseudotype viruses produced in the presence of hA3G was determined by infecting 293 cells. The number of cells expressing β-galactosidase was evaluated by X-gal staining for virus infectivity. The infectivity of the pseudotype viruses produced in empty-vector-transfected cells was set to 100%. T-hu3G: transient hA3G-expressing 293 cells. S-hu3G: stable hA3G-expressing 293 cells.

assay (Fig. 3). To investigate whether hA3G and human tetherin inhibit PERV replication in a spreading infection as efficiently as in the pseudotype assay, virus spread was monitored by real-time RT-PCR. The expression plasmids for hA3G and human tetherin were transfected into 293-PERV-PK-CIRCE cells. In agreement with the pseudotype assay, hA3G and human tetherin inhibited PERV replication in a spreading infection (Fig. 4). In contrast to the marked inhibitory effect of hA3G in spreading infection, tetherin showed a weaker antiviral activity.

Previous studies have reported a dose-dependent relationship between A3G levels and virus infectivity [22]. To compare the antiviral effect between stable 293 cells expressing hA3G, and 293T cells transiently expressing hA3G, we constructed a cell line that stably expressed hA3G. The expression level of hA3G in the stable 293 cells was higher than that in the 293T cells transiently transfected with increasing amounts of hA3G (Fig. 5). Fig. 6 shows that viruses from the 293 cells stably expressing hA3G have little infectivity. This finding suggests that viruses from the 293 cells stably expressing hA3G contain more hA3G than transiently transfected cells. Thus, severe overexpression of hA3G may be critical for hA3G antiviral activity.

In this study, we examined a comparative analysis of hA3G and human tetherin. hA3G was the most potent infection inhibitor, and reduction in infectivity was directly proportional to the amounts of virus-associated hA3G. In conclusion, a combination of hA3G and human tetherin may reduce PERV dissemination in xenotransplantation.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0024912).

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