Canine ASCT1 and ASCT2 are functional receptors for RD-114 virus in dogs

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All domestic cats carry an infectious endogenous retrovirus termed RD-114 virus. Recently, we and others found that several live-attenuated vaccines for dogs were contaminated with infectious RD-114 virus. In this study, we confirmed that the RD-114 virus efficiently infected and proliferated well in canine primary kidney cells, as well as three tested canine cell lines. Further, we identified canine ASCT1 and ASCT2, sodium-dependent neutral amino acid transporters, as RD-114 virus receptors. Canine ASCT2 also acts as a functional receptor for simian retrovirus 2, a pathogenic retrovirus that induces immunodeficiency in rhesus macaques. Identification of the canine receptor for RD-114 virus will help in evaluating the risk from vaccines contaminated by the virus.

All domestic cats carry an infectious endogenous retrovirus termed RD-114 virus (Fischinger et al., 1973; Okabe et al., 1973; Baumann et al., 1998). Several feline cell lines, such as Crandell–Rees feline kidney (CRFK) cells, produce RD-114 virus (Baumann et al., 1998; Yoshikawa et al., 2010; Okada et al., 2011), and most live-attenuated vaccines for dogs and cats are produced using feline cell lines. Recently, we found that several live-attenuated vaccines produced using feline cell lines were contaminated with infectious RD-114 virus (Miyazawa et al., 2010). Furthermore, we found that several canine live-attenuated vaccines produced using non-feline cell lines were also contaminated with infectious RD-114 virus (Yoshikawa et al., 2011a). In this case, a seed stock of canine parvovirus that had been isolated and attenuated in feline cells was potentially contaminated with infectious RD-114 virus. In fact, we confirmed that a proportion of canine parvovirus stocks that had been isolated and propagated in feline cells were contaminated with infectious RD-114 virus (Yoshikawa et al., 2011b).

It is important to identify virus receptor(s) to know more about virus tropisms and pathogenesis. RD-114 virus is known to infect non-feline cell lines, including human and canine cell lines (Roth et al., 1983; Takeuchi et al., 1992, 1994, 1996; Baumann et al., 1998). In human cell lines, RD-114 virus interferes with baboon endogenous retrovirus (BaEV), simian retroviruses (SRV) 1, 2, 3, 4 and 5, avian reticuloendotheliosis virus and duck spleen necrosis virus (Rasko et al., 1999; Tailor et al., 1999); therefore, these retroviruses are considered to utilize the same receptor in human cells. The human receptor for RD-114 virus is a sodium-dependent neutral amino acid transporter termed ASCT (Marin et al., 2000). Both mice and humans have two types of ASCT molecule, termed ASCT1 and ASCT2. The similarity between ASCT1 and ASCT2 is approximately 57%, and the amino acids transported by ASCT1 and ASCT2 are not identical (Arriza et al., 1993; Utsunomiya-Tate et al., 1996). RD-114 virus utilizes both human ASCT1 and ASCT2, but the virus uses ASCT2 more efficiently than ASCT1 (Marin et al., 2003). RD-114 virus does not infect murine NIH3T3 cells; however, the virus did infect these cells when they were treated with tunicamycin (Marin et al., 2000). BaEV infects murine cells (NIH3T3 cells), but it utilizes ASCT1 instead of ASCT2 to infect these cells (Marin et al., 2000). Although RD-114 virus infects canine cell lines, there has been no report on the receptor for RD-114 virus in dogs. In this study, we found that RD-114 virus productively infects primary canine cells, and report that canine ASCT1 and ASCT2 function as the RD-114 receptor.

TE671 cells (human rhabdomyosarcoma) (Stratton et al., 1989), TELCeB/AF-7 cells (Cosset et al., 1995), TELCeB cells persistently infected with SRV-2 or BaEV (TELCeB/
SRV-2 or TELCeB/BaEV cells; Tailor et al., 1999), Cf2Th cells (a fibroblast cell line derived from thymus) (CRL-1430; ATCC), D17 cells (a canine cell line derived from osteosarcoma) (CCL-183; ATCC), Madin–Darby canine kidney (MDCK) cells (CCL-34; ATCC), DK/P cells (primary epithelial cells derived from canine kidney), Mus dunnii tall fibroblast (MDTF) cells, G355-5 cells (feline glial cells) and CRFK cells (CCL-94; ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, penicillin (100 U ml\(^{-1}\)) and streptomycin (100 \(\mu\)g ml\(^{-1}\)) (Invitrogen). G355-5, Cf2Th and DK/P cells transduced with the \text{nlslacZ} gene [G355-5(lacZ), Cf2Th(lacZ) and DK/P(lacZ) cells, respectively], and G355-5 and G355-5(lacZ) cells inoculated with a supernatant of CRFK cells [G355-5(CRsup and G355-5(lacZ)/CRsup, respectively], were also cultured in DMEM.

The \text{nlslacZ} gene has a nuclear-localization signal upstream of the ORF of the lacZ gene.

RD-114 virus was prepared from culture supernatant of G355-5/CRsup cells. lacZ(A-MLV) virus, with an envelope of amphotropic murine leukemia virus (A-MLV), core of MLV and the \text{nlslacZ} gene, was prepared from TELCeB/BaEV cells, lacZ(RD-114) virus, with an envelope of RD-114 virus, core of RD-114 virus and the \text{nlslacZ} gene, was prepared from G355-5(lacZ)/CRsup cells. lacZ(SRV-2) virus, with an envelope of SRV-2, core of MLV and the \text{nlslacZ} gene, was prepared from TELCeB/SRV-2 cells. lacZ(BaEV) virus, with an envelope of BaEV, core of MLV and the \text{nlslacZ} gene, was prepared from TELCeB/BaEV cells. All stock viruses were filtered through a 0.45 \(\mu\)m membrane filter (PALL) and kept at 80\(^\circ\) C until used. The titre of the stock virus of RD-114 virus was determined by the S+L– focus assay using QN10S cells as described previously (Sakaguchi et al., 2008a).

To confirm whether functional receptors for RD-114 virus are expressed in various canine cells, either lacZ(RD-114) or lacZ(A-MLV) virus was inoculated into canine, murine and human cells as indicated in Fig. 1(a). Target cells were seeded in 24-well plates at 8 \times 10^4 cells per well 1 day before infection. Virus inoculation was performed at 37 °C by plating 500 \(\mu\)l serially diluted lacZ(RD-114) or lacZ(A-MLV) virus into target cells in the presence of 8 \(\mu\)g polybrene (hexadimethrine bromide) ml\(^{-1}\) (Sigma-Aldrich). Two days after inoculation, all inoculated cells were stained using X-Gal, and virus titres, expressed as blue focus-forming units (f.f.u.) ml\(^{-1}\), were determined (Sakaguchi et al., 2008b). As a result, lacZ(A-MLV) virus efficiently infected all cells tested, and lacZ(RD-114) virus efficiently infected all canine and human cells tested, but not mouse MDTF cells (Fig. 1a).

To determine whether canine cells support the replication of RD-114 virus, RD-114 virus was inoculated into DK/P(lacZ) cells (primary canine kidney cells) and Cf2Th (LacZ) cells (a canine fibroblast cell line derived from thymus) Target cells were seeded in T25 culture flasks at 10^5 cells per flask. RD-114 virus (1000 f.f.u. in 1 ml) was inoculated into the cells in the presence of 8 \(\mu\)g polybrene ml\(^{-1}\) and then incubated at 37 °C for 4 h for virus adsorption. Culture supernatants were collected at 3, 7, 11, 15, 19 and 23 days after inoculation. The growth kinetics of RD-114 virus were determined by LacZ marker rescue assay (Sakaguchi et al., 2008b). Fifteen days after inoculation, the titre of RD-114 virus reached a plateau in both cell types (Fig. 1b). The maximum titre of RD-114 virus produced in DK/P(LacZ) cells was 1.17 \times 10^5 f.f.u. ml\(^{-1}\).

Next, we attempted to clone canine ASCT1 and ASCT2 cDNA by RT-PCR. Canine RNA was prepared from Cf2Th cells with an RNeasy mini kit (Qiagen). cDNA was synthesized from 450 ng RNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). To clone canine ASCT1 and ASCT2 cDNA, Cf2Th cDNAs were amplified using primers corresponding to canine ASCT1 (forward primer, 5'-ATGGATGGTGGCCGATCCGCCC-3'; reverse primer, 5'-TTACATAACTGATTCCTTCTC-3') and canine ASCT2 (forward primer, 5'-ATGGTGGCCGATCCGCCC-3'; reverse primer, 5'-TTACATAACTGATTCCTTCTCCGAGGGTT-3'). PCR conditions were as follows: the reaction mixture (total 50 \(\mu\)l) consisted of 3 \(\mu\)l cDNA template, 10 \(\mu\)l 5× buffer containing 5 mM MgCl_2 (PrimeSTAR GXL buffer; TaKaRa), 2 \(\mu\)l PrimeSTAR GXL DNA polymerase, 0.2 \(\mu\)mol of each primer, and nuclease-free water up to 50 \(\mu\)l.

**Fig. 1.** Susceptibility of canine cells to RD-114 virus. (a) Infectivity of lacZ(RD-114) and lacZ(A-MLV) viruses to DK/P, Cf2Th, MDCK, D17, MDTF and TE671 cells. Grey- and white-filled bars represent the titres of lacZ(RD-114) and lacZ(A-MLV) viruses, respectively. (b) Growth of RD-114 virus in canine cells. RD-114 virus released in the culture supernatants was monitored by LacZ marker rescue assay. Solid (■) and dotted (●) lines represent Cf2Th(LacZ) and DK/P(LacZ) cells, respectively.
polymerase (TaKaRa), 4 μl 2.5 mM dNTPs, 1 μl of each primer (10 μM) and 28 μl distilled water. The amplification conditions for both canine ASCT1 and ASCT2 were 98 °C for 5 min followed by 30 cycles of amplification, consisting of denaturation at 98 °C for 10 s, annealing at 58 °C for 15 s and extension at 68 °C for 16 s, and then final extension at 68 °C for 5 min. PCR was carried out in 200 μl thin-walled tubes using a C1000 Thermal Cycler (Bio-Rad). The amplicons, corresponding to the entire canine ASCT1 and ASCT2 genes, were inserted into pACGFP-N1 (Clontech) to produce pcaASCT1/GFP and pcaASCT2/GFP, respectively. The amino acid sequences of the cloned canine ASCT1 and ASCT2 were identical to those deposited in GenBank for canine ASCT1 (accession no. XP_538511) and ASCT2 (accession no. XP_541540), respectively.

To express ASCT molecules, pcaASCT1/GFP, pcaASCT2/GFP or pACGFP-N1 was transfected into MDTF cells using ExGen 500 in vitro Transfection Reagent (Fermentas) according to the manufacturer’s instructions. One day after transfection, the expression of GFP was observed with a fluorescence microscope (IX72 UV microscope; Olympus). Consequently, the expression of both canine ASCT1–GFP and ASCT2–GFP fusion proteins was confirmed in MDTF cells (Fig. 2a).

To confirm whether canine ASCT1 and ASCT2 function as RD-114 receptors, MDTF cells expressing canine ASCT1–GFP, canine ASCT2–GFP or GFP (MDTF/caASCT1, MDTF/caASCT2 or MDTF/GFP cells) or Cf2Th cells were seeded in 96-well plates at 1 × 10^4 cells per well 1 day before infection. Virus inoculation was performed at 37 °C by plating 100 μl serially diluted lacZ(RD-114) virus into target cells in the presence of 8 μg polybrene ml⁻¹ (Sigma-Aldrich). Two days after inoculation, the inoculated cells were stained using X-Gal, and virus titres, expressed as f.f.u. ml⁻¹, were determined (Sakaguchi et al., 2008b). As a result, lacZ(RD-114) virus infected MDTF/caASCT1, MDTF/caASCT2 and Cf2Th cells (Fig. 2b, c); however, lacZ(RD-114) virus infected MDTF/caASCT2 cells more efficiently than MDTF/caASCT1 cells (Fig. 2b, c). From these data, we conclude that both canine ASCT1 and ASCT2 function as RD-114 receptors.

In human cells, human ASCT2 functions as receptor for SRV-2 and BaEV (Tailor et al., 1999). To confirm whether canine ASCT1 and ASCT2 function as SRV-2 and BaEV receptors, lacZ(SRV-2) and lacZ(BaEV) viruses were inoculated into MDTF/GFP, MDTF/caASCT1, MDTF/caASCT2 or Cf2Th cells. Two days after inoculation, the inoculated cells were stained using X-Gal, and virus titres, expressed as f.f.u. ml⁻¹, were determined (Sakaguchi et al., 2008b). Consequently, we found that lacZ(SRV-2) virus infected MDTF/caASCT2 and Cf2Th cells, but not MDTF/caASCT1 cells (Fig. 2b), indicating that canine ASCT2, but not ASCT1, functions as an SRV-2 receptor. In addition, lacZ(BaEV) virus infected MDTF/GFP, MDTF/caASCT1, MDTF/caASCT2 and Cf2Th cells (Fig. 2b). However, lacZ(BaEV) virus infected MDTF/caASCT1 and MDTF/caASCT2 cells more efficiently than MDTF/GFP cells (Fig. 2b), suggesting that both canine ASCT1 and ASCT2 function as BaEV receptors.

In previous studies, we reported that several canine and feline attenuated vaccines were contaminated with infectious...
RD-114 virus (Miyazawa et al., 2010; Yoshikawa et al., 2010, 2011a). The Japanese regulatory authority also confirmed this incidence independently (Narushima et al., 2010, 2011b). In a certain canine vaccine, the amount of contaminated RD-114 virus was rather high, reaching 1.35 million copies per dose (Narushima et al., 2011b) and 10,000 TCID50 per dose (Yoshikawa et al., 2011a). In this study, we confirmed that the RD-114 virus efficiently infected and proliferated well in canine primary cells. Together with a report by Ting-De Ravin et al. (2006), which demonstrated that a foreign gene (interleukin-2 receptor γ chain) was delivered to canine bone-marrow cells when dogs were inoculated intravenously with RD-114-pseudotyped retrovirus vector, we consider that the possibility that RD-114 virus infects dogs in vivo cannot be excluded.

In contrast to our supposition, Narushima et al. (2011a) reported that RD-114 proviral DNA was not found in dogs inoculated subcutaneously with RD-114 virus. However, they only investigated RD-114 provirus in quite limited tissues (lymph nodes, spleen and bone marrow) and peripheral blood, and the sensitivity of the one-step PCR to detect RD-114 proviral DNA was obscure. Here, we demonstrate that both canine ASCT1 and ASCT2 function as receptors for RD-114 virus, but RD-114 virus utilizes ASCT2 more efficiently than ASCT1. In humans, ASCT1 is expressed ubiquitously in tissues (Arriza et al., 1993), whereas the expression of ASCT2 was not detected in various tissues, and the expression level of ASCT2 also varies among tissues (Utsunomiya-Tate et al., 1996; Green et al., 2004). Because the distribution of ASCT1 and ASCT2 in dogs is unknown at present, the principal target tissues of RD-114 virus cannot be predicted.

In human cells, RD-114 virus shares its receptor with pathogenic retroviruses, such as SRVs, avian reticuloendotheliosis virus and duck spleen necrosis virus. In particular, SRV-1, SRV-2 and SRV-3 are known to induce fatal immuno-deficiency in rhesus macaques, termed simian acquired immunodeficiency syndrome (Marx et al., 1984, 1985; Montiel, 2010). In this study, we also demonstrated that SRV-2 utilizes canine ASCT2 as receptor (Fig. 2b). Intriguingly, RD-114 virus has an immunosuppressive domain in its transmembrane envelope protein, and the amino acid sequence of this domain (LQNRRGLDLTAEQGGI) is identical to those of SRV-1, SRV-2 and SRV-3 (Cianciolo et al., 1985; Blaise et al., 2001). Therefore, RD-114 virus may induce immunosuppression, if it has adapted to grow efficiently in dogs.

At present, the real risk from contamination of RD-114 virus in canine vaccines is still unknown. Identification of the canine receptor for RD-114 virus will shed light on evaluating the risk from vaccines contaminated by the virus.

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