Characterization of feline ASCT1 and ASCT2 as RD-114 virus receptor

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RD-114 virus is a replication-competent feline endogenous retrovirus (ERV). RD-114 virus had been thought to be xenotropic; however, recent findings indicate that RD-114 virus is polytropic and can infect and grow efficiently in feline cells. Receptor(s) for RD-114 virus have not been identified and characterized in cats. In this study, we confirmed that two feline sodium-dependent neutral amino acid transporters (ASCTs), fASCT1 and fASCT2, function as RD-114 virus receptors. By chimeric analyses of feline and murine ASCTs, we revealed that extracellular loop 2 of both fASCT1 and fASCT2 determines the susceptibility to RD-114 virus. Further, we revealed ubiquitous expression of these genes, consistent with the general metabolic role of the ASCT molecules. Our study indicates that RD-114 virus may re infect tissues and cells in cats, once the virus is activated. Implications of the involvement of RD-114 virus in feline oncogenesis are also discussed.

Endogenous retroviruses (ERVs) are retroviruses that have been integrated into the host genome of germ-line cells and comprise approximately 10% of the host genome in mammals (Gifford & Tristem, 2003). ERVs behave as the host’s genes and are transmitted from parent to offspring by Mendelian laws of inheritance. Although many ERVs are inactivated through the accumulation of mutations, deletions and the introduction of termination signals within coding sequences, some ERVs are active and have the potential to produce infectious viral particles. Most ERVs are thought not to be involved in any diseases; however, there are several incidences where ERVs exhibit pathogenicity. In AKR mice, an infectious endogenous murine leukaemia virus (MLV) in the genome is activated during development and induces lymphoma (Hartley et al., 1977; McGrath & Weissman, 1979). Recently, it was found that replication activity of mouse ERVs was resurrected in recombinase-activating gene knockout mice, which had no mature B- and T-lymphocytes, and Toll-like receptor (TLR) 3, 7 and 9 triple deficient mice (Young et al., 2012; Yu et al., 2012). In addition, activated ERVs in the TLR-knockout mice induced lymphoma in the host (Yu et al., 2012). These reports suggest that infectious ERVs can exhibit oncogenicity in the host. In these cases, receptors for infectious ERVs were expressed in tissues of mice and infectious ERVs reinfected the host cells by using these receptors to induce lymphoma, possibly via insertion of the ERVs in the vicinity of cellular proto-oncogenes.

RD-114 virus is a replication-competent feline ERV (Reeves & O’Brien, 1984), and several feline cell lines produce RD-114 virus. RD-114 virus has been classified as a xenotropic virus which cannot infect the original host (Fischinger et al., 1973; Teich, 1984). Thus, it had been considered that even if RD-114 virus is activated in cats, RD-114 virus cannot reinfect tissues and cells in cats. However, we and others reported that several feline cell lines are susceptible to RD-114 virus (Dunn et al., 1993; Okada et al., 2011); therefore, RD-114 virus is now considered to be polytropic and can infect both the original host species (i.e. cats) and other species (Okada et al., 2011). The receptors for viruses which interfere with RD-114 virus (i.e. the same interference group) have been identified as sodium-dependent neutral amino acid transporter (ASCT) types 1 and 2 (ASCT1 and ASCT2, respectively) in humans, rats, mice and dogs (Marin et al., 2000; Rasko et al., 1999; Tailor et al., 1999; Yoshikawa et al., 2012b), but ASCT molecules as the RD-114 virus receptor in cats have not been characterized. In this study, to understand the pathogenic potential of RD-114 virus in cats, we identified feline ASCT1 (fASCT1) and ASCT2 (fASCT2) as RD-114 virus receptors and characterized them.
As CT2 (data not shown). Hydropathy were similar to those of human ASCT1 and 952 (domain 7) and a long hydrophobic stretch. These patterns of 954 hydropathy revealed that the proteins contained six prominent hydro- 963 phobic domains (domains 1–6), a small hydrophobic peak 965 (domain 7) and a long hydrophobic stretch. These patterns of 967 hydropathy were similar to those of human ASCT1 and 968 ASCT2 (data not shown).

The sequence of the fASCT2 gene showed 85, 79, 78 and 91 % identity with human, mouse, rat and dog ASCT2, respectively. The sequence of the fASCT1 gene showed 90, 84, 83 and 93 % identity with human, mouse, rat and dog ASCT1, respectively. The open reading frames of the fASCT1 and fASCT2 genes were 1599 and 1626 bp long, encoding predicted 532 and 541 aa, respectively. Only the adenine at nucleotide position 132 of predicted fASCT1 mRNA was substituted to cytosine in our cloned fASCT1 cDNA, and the amino acid sequences of both fASCT1 and fASCT2 (GenBank accession numbers AB794688 and AB794689, respectively) were identical to the predicted fASCT mRNA sequences already deposited (fASCT1, XM_003984056.1; fASCT2, XM_003997623.1). Identity between fASCT1 and fASCT2 amino acid sequences was 58 %. The amino acid sequence of the fASCT1 gene showed 90, 84, 83 and 93 % identity with human, mouse, rat and dog ASCT1, respectively, and the sequence of the fASCT2 gene showed 85, 79, 78 and 91 % identity with human, mouse, rat and dog ASCT2, respectively (Table 1). As shown in Fig. S1 (available in JGV Online), Kyte–Doolittle hydropathy analysis (Kyte & Doolittle, 1982) revealed that the proteins contained six prominent hydrophobic domains (domains 1–6), a small hydrophobic peak (domain 7) and a long hydrophobic stretch. These patterns of hydropathy were similar to those of human ASCT1 and ASCT2 (data not shown).

To investigate the function of fASCT1 and fASCT2 as RD-114 virus receptors, pfASCT1/GFP, pfASCT2/GFP or pACGFP-N1 (the empty vector) was transfected into Mus dunni tail fibroblast (MDTF) cells (not susceptible to RD-114 virus) using Lipofectamine 2000 transfection reagent (Invitrogen) 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 fASCT1- and fASCT2-GFP fusion proteins was confirmed in the transfected MDTF cells (Fig. 1b). To confirm whether fASCT1 and fASCT2 function as RD-114 receptors, MDTF cells expressing fASCT1-GFP, fASCT2-GFP or GFP (the empty vector) (MDTF/fASCT1, MDTF/ fASCT2 or MDTF/GFP cells, respectively) or G355-5 cells were seeded in 96-well plates at 1 × 10^4 cells per well 1 day before infection. lacZ(RD-114) pseudotype virus, having an envelope of RD-114 virus, core of MLV and the nls-lacZ gene, was prepared from G355-5(LacZ)/CR cells (Yoshikawa et al., 2012a). Virus inoculation was performed at 37 °C by placing 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.u. ml^{-1}, were determined as described previously (Sakaguchi et al., 2008; Takeuchi et al., 1998).

lacZ(RD-114) virus infected MDTF/fASCT1, MDTF/ fASCT2 and G355-5 cells, but did not infect MDTF cells expressing only GFP by the empty vector (Fig. 1c). lacZ(RD-114) virus infected MDTF/fASCT2 cells more efficiently than MDTF/fASCT1 cells (Fig. 1c, d). In this expression system, FASCT2 was expressed in larger amounts than FASCT1 (data not shown), which may explain the difference in virus susceptibility between fASCT1 and fASCT2. From these results, we concluded that both fASCT1 and fASCT2 function as RD-114 virus receptors.

RD-114 virus does not use mouse ASCT1 or ASCT2 as a receptor (Koo et al., 1994; Marin et al., 2000). By using feline–mouse ASCT chimera, we tried to identify the sites of fASCT1 and fASCT2 that determine the susceptibility of the cells to RD-114 virus. We targeted extracellular loop 2 (ECL2) of the ASCTs as the site determining the susceptibility to RD-114 virus because Marin et al. (2003) reported that the ECL2 is where glycosylation of N-linked oligosaccharides occurs in human ASCT1 and ASCT2 and ECL2 plays critical roles in controlling reception of viruses such as RD-114 virus, baboon endogenous retrovirus (BaEV) and type D primate retroviruses (simian beta-retroviruses). Feline–mouse ASCT1 and ASCT2 chimeric constructs were made by using the primers listed in Table S1 with an In-Fusion HD cloning kit (Clontech) according to the manufacturer’s instructions. Primers were designed to amplify ECL2 (fE/mASCT1, mE/fASCT1, mE/mASCT2 and mE/fASCT2) and the carboxyl-terminal regions in ECL2 (termed Region C; fC/mASCT1 and mC/fASCT2). The cDNAs of mouse ASCT1 and ASCT2 (mouse cDNA clones SLCLA4 and SLCLA5, respectively) were purchased...
from Origine. Feline–mouse ASCT1 and ASCT2 chimeric cDNAs (Fig. 1a) were inserted into pACGFP1-N1 and transfected into MDTF cells as described above. One day after transfection, the expression of GFP was confirmed and MDTF cells expressing feline–mouse ASCT chimeras or G355-5 cells were seeded in 96-well plates one day before virus infection. Virus inoculation was performed as described above. 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., 2008; Takeuchi et al., 1998). We found that Region C in the ECL2 of both fASCT1 and fASCT2 plays critical roles in controlling RD-114 virus reception (Fig. 1). Many retroviruses recognize conserved regions that are important for the physiological function of the receptor, but Region C is richly diverse among species (Fig. S1). RD-114 virus, BaEV and type D primate retroviruses, which have distinct host ranges, use human ASCT2 as a common receptor. RD-114 virus and type D primate retroviruses were able to infect murine cells only after these cells were treated with tunicamycin, an inhibitor of protein N-linked glycosylation (Koo et al., 1994; Marin et al., 2000). Based on the tunicamycin-dependent susceptibility of murine cells to the retroviruses and our feline–mouse ASCT chimera assay, we suspected that N-linked glycosylation in ECL2, especially Region C, in mASCTs might contribute to blocking access of viruses to an essential recognition (or binding) site in the receptor of mice.

Finally, we investigated expression patterns of fASCT1 and fASCT2 in multiple tissues of domestic cats. Cellular RNA (100 ng) extracted from various tissues of cats ($n=3$) with an RNase Mini kit was reverse transcribed with SuperScriptIII reverse transcriptase according to the manufacturer’s instructions. Real-time PCR was carried out in a 20 µl reaction mixture using SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer’s instructions with an Applied Biosystems 7000 real-time PCR system. A negative control with no

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**Fig. 1.** Functional assay of fASCT1, fASCT2 and feline–mouse ASCT chimeras as RD-114 virus receptors. (a) Construction of fASCT1/mASCT1 and fASCT2/mASCT2 chimeras. (b) Expression of feline ASCT1-GFP, ASCT2-GFP, feline–mouse ASCT chimeras-GFP and GFP in MDTF cells detected by fluorescence microscopy. (c, d) lacZ pseudotype virus infection assay was performed in MDTF cells transiently expressing fASCT1, fASCT2 and feline–mouse ASCT chimeras. Infection with lacZ(RD-114) pseudotype virus was visualized by X-Gal staining (c) and the virus titres were expressed as f.f.u. ml$^{-1}$ (d).
template was also included. Primers (fASCT1 ex-forward primer, 5′-CACAACAGGAGCTTGAAA-3′ and ex-reverse primer, 5′-AGGCATTGAAGAAACGGATG-3′; fASCT2 ex-forward primer, 5′-AGCCAGATGATCAGGAAAC-3′ and ex-reverse primer, 5′-ATGATCCAGGACGAGGAC-3′) to quantify the mRNA of fASCT1 and fASCT2, respectively, were designed to span an exon/intron junction of the gene to avoid amplification of DNA sequences, using Primer3 software in GENETYX ver.11 (GENETYX). Feline β-actin primers (fACTB F1 and R1), designed based on a previous report (Kessler et al., 2009), were used as an internal control. Consequently, real-time RT-PCR showed that both fASCT1 and fASCT2 are expressed in most tissues in cats (Fig. 2). Relatively high expression levels of fASCT1 and fASCT2 were observed in pancreas, cerebrum and cerebellum, and submandibular gland, kidney, pancreas and bone marrow, respectively (Fig. 2). Expression patterns of fASCT1 and fASCT2 were basically similar to those of other mammalian ASCT1 and ASCT2 genes (Arriza et al., 1993; Utsunomiya-Tate et al., 1996). Highest expression of fASCT2 in bone marrow indicates that RD-114 virus may infect haematopoietic cells efficiently.

Recently, it was reported that in immune-compromised mice, ERVs in the host’s genome were resurrected and induced lymphoma (Young et al., 2012; Yu et al., 2012). It is still unknown whether RD-114 virus causes diseases; our recent data together with the current study indicate that RD-114 virus may reactivate a wide variety of feline tissues in vivo, once infectious RD-114 virus is activated in cats. Thus, it is possible that RD-114 virus induces neoplastic diseases if the provirus is integrated in the vicinity of proto-oncogenes. In veterinary practices, feline leukaemia virus (FeLV) is considered to be the primary cause of many types of lymphoma in cats; however, recent data indicate that many aged cats develop non-FeLV-type lymphoma (Louwerens et al., 2005; Teske et al., 2002). In recent decades, although FeLV testing, elimination programmes and vaccinations have reduced the subsequent FeLV infection rates significantly, the incidence of lymphoma in cats has increased. Teske et al. (2002) tested 54 cats with malignant lymphoma at the Utrecht University Clinic for Companion Animals from 1984 to 2000 and the Referral Specialist Clinic Amsterdam from 1997 to 2000, and revealed that 50 (92.6 %) were non-FeLV-type lymphoma. Louwerens et al. (2005) reported that from 1983 to 2003, 467 (85.5 %) of 546 cases of feline lymphoma at the Veterinary Medicine Teaching Hospital in California were non-FeLV-type lymphoma. In the context of the possible involvement of RD-114 virus in lymphoma, it was reported that RD-114 virus was highly expressed in spontaneous lymphoma (Niman et al., 1977) and a large granular lymphoma cell line, MCC cells, produces infectious RD-114 virus (Cheney et al., 1990; Okada et al., 2011). Therefore, we suspect that RD-114 virus is involved, in part, in generative diseases in cats. In addition, the findings that cats express functional receptors in many tissues may cast another concern about the implication of live attenuated vaccines contaminated with infectious RD-114 virus reported recently (Miyazawa, 2010; Miyazawa et al., 2010; Narushima et al., 2010, 2011; Yoshikawa et al., 2010, 2011). Our findings reported here will be useful for further analyses to reveal the potential pathogenicity of RD-114 virus in cats.

![Fig. 2. Expression of fASCT1 and fASCT2 in multiple tissues in cats (n=3). Sk, Skin; Sg, submandibular gland; Mln, mandibular lymph node; To, tonsils; Thym, thymus; Thy, thyroid; He, heart; Lu, lung; Li, liver; Sp, spleen; Ki, kidney; Ad, adrenal gland; Te, testis; Prg, prostate gland; St, stomach; Pa, pancreas; Du, duodenum; Re, rectum; Bl, bladder; Bm, bone marrow; Cbel, cerebellum; Cbr, cerebrum. Each value was normalized to the amount of ACTB mRNA and expressed as the mean ± SEM of data from three samples in triplicate experiments.](http://vir.sgmjournals.org)
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References


