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Short Communication

CXCR4 is the primary receptor for feline immunodeficiency virus in astrocytes

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Feline astrocytes were productively infected with the Crandell feline kidney (CrFK) cell-adapted feline immunodeficiency virus (FIV) Petaluma strain in a primary culture. They expressed mRNA of CXCR4, and the FIV infection was blocked by stromal cell-derived factor 1α (SDF-1α), SDF-1β, or the bicyclam AMD3100 in a dose-dependent manner. These observations suggest that, like FIV infection in CrFK cells and lymphocytes, the virus uses CXCR4 as a primary receptor for infecting astrocytes and this can be a possible natural model for AIDS dementia complex. *Journal of NeuroVirology* (2001) **7,** 487–492.

Keywords: FIV; chemokine receptor; AMD3100; astrocytes

Introduction

The human central nervous system is a target of the human immunodeficiency virus type-1 (HIV-1), which results in the AIDS dementia complex (ADC) (Lipton and Gendelman, 1995). It has long been suggested, based on the finding of the productive viral infection in microglia with CD4 (Jordan *et al*, 1991; Watkins *et al*, 1990), that neuronal pathology in ADC is possibly caused by various proinflammatory cytokines and/or toxic substances produced by activated microglia (Genis *et al*, 1992; Dowson *et al*, 1993; Kerr *et al*, 1998). However, it still remains unclear whether the cytokines and/or the toxic substances can, in fact, induce neuronal damage. Meanwhile, HIV-1 has been suggested to utilize a receptor other than CD4 by demonstration of the presence of viral proteins or genomes in astrocytes and neurons (Wiley *et al*, 1986; Gyorkey *et al*, 1987) that do not have a CD4 molecule.

Feline immunodeficiency virus (FIV), a member of the lentivirus family of retroviruses, was isolated from domestic cats with AIDS-like syndrome such as severe immunodeficiency and neurological impairment (Pedersen *et al*, 1987). Neurological damage induced by FIV infection includes behavioral changes, electrophysiological abnormalities, sleep disturbances, and seizures (Pedersen *et al*, 1987; Dow *et al*, 1990; Phillips *et al*, 1994; Prospero-Garcia *et al*, 1994). FIV is also known to cause perivascular infiltration, gliosis, white matter pallor (Dow *et al*, 1990; Hurtrel *et al*, 1992; Phillips *et al*, 1994), and neuronal damage (Gruol *et al*, 1998) or loss (Power *et al*, 1997). Although FIV infects astrocytes and microglia/macrophages *in vitro* (Dow *et al*, 1990), the exact neuropathogenic mechanisms are still unclear. FIV has been known to involve a mode of infection that does not utilize the CD4 molecule as a primary receptor, in spite of being T lymphotropic virus. This is due not only the fact that the virus infects various kinds of CD4(−) cells, including feline fibroblasts (Yamamoto *et al*, 1988), astrocytes (Dow *et al*, 1990), and CD8(+) lymphocytes (Brown *et al*, 1991), but also because the infection cannot be inhibited by antibodies against CD4 (Hosie *et al*, 1993). Further inquiries into the FIV receptor still need to be carried out to obtain a deeper understanding of the mechanisms involved.

CXCR4, a chemokine receptor, is a member of the G protein-coupled molecules with 7 transmembrane domains, and it mediates the chemotaxis of T cells (MacKay, 1996). In 1996, the molecule was first identified as a coreceptor for HIV-1 entry into helper T cells, in combination with a CD4 molecule (Feng

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et al, 1996). In addition, CXCR4 has been shown to function as a primary receptor for HIV-2 infection in lymphocytes (Endres *et al*, 1996) and FIV infection in CrFK (Willett *et al*, 1997; Hosie *et al*, 1998; Richardson *et al*, 1999) and lymphocytes (Richardson *et al*, 1999). These infections could be blocked by a biological or by artificial ligands for CXCR4 such as SDF-1 (Hosie *et al*, 1998) and bicyclam derivatives (De Clercq *et al*, 1992; Egberink *et al*, 1999; Richardson *et al*, 1999), respectively. The fact that CXCR4 molecules are expressed in various types of cells including neurons, astrocytes, and microglia (Lavi *et al*, 1997; Tanabe *et al*, 1997; Ohtani *et al*, 1998; Klein *et al*, 1999) in the brain leads to the attractive hypothesis that these molecules may also function as receptor for HIV-1 and FIV infection in neural cells. Recent studies have revealed that the blood-derived CXCR4-dependent HIV-1 induces apoptosis of neurons 3- to 8-fold greater than the macrophage(M)-tropic HIV-1 (Ohagen *et al*, 1999). In this work, we stress the importance of CXCR4 in the FIV infection of feline astrocytes by showing CXCR4 expression in astrocytes and blocking the infection with SDF-1α, SDF-1β, or the bicyclam, AMD3100, and discuss the potential therapeutic possibilities.

First, using a RT-PCR technique, we determined whether CXCR4 is expressed on astrocytes and microglia, which are target cells of FIV. Feline astrocytes or microglia were dissociated from the specific pathogen-free feline brains at 7 or 8 days of age by 60-min incubation in HBSS containing papain at 37°C. Subsequently, single cells were passed through a 100-μm nylon mesh and cultured in Iscove's Modified Dulbecco MEM (IMDM, Gibco) and fetal bovine serum (Gibco). After 10 days, microglia-rich cultures were obtained by the adhesion of floating microglia onto an uncoated plastic dish. Astrocyte-rich cultures were prepared by the elimination of microglia with trypsinization three times. Both cultures showed more than 98% purity, as determined by immunohistochemistry with polyclonal antibody to glial fibrillary acidic protein (DAKO), and isolectin (Vector Lab) as markers specific for astrocytes and microglia, respectively.

Total cellular RNA was extracted with a Quickprep micro mRNA purification kit (Pharmacia) from cultured microglia or astrocytes, and cDNA was synthesized with a first-strand cDNA synthesis kit (Pharmacia) according to the manufacturer's instructions. The oligonucleotide primers (sense: GACCGCTTCTATCCCAGTGA; antisense: CAGTGGAAAAAGGCTAGGGC) of the feline CXCR4 were constructed based on the registered sequence in GenBank (U63558). PCR was performed, using a thermal cycler (TaKaRa) and Ex taq DNA polymerase (TaKaRa). The profile of the thermal cycle was 1) denaturation at 93°C for 20 s, 2) annealing at 60°C for 30 s and 3) extension at 72°C for 45 s. The PCR products obtained by 30 cycles were electrophoresed on a 6% acrylamide gel and stained with ethidium bromide. Additionally, to confirm the sequence of CXCR4, the amplified products by PCR were digested by Sau 3AI (TOYOBO) for 1.5 h at 37°C, and electrophoresed on an 8% acrylamide gel. The PCR products from both the cultured feline astrocytes and microglia migrated to the expected position (325 bp) of feline CXCR4, as in the case of feline lymphocytes and CrFK (Figure 1a). The application of a restriction enzyme ensured that the PCR products were of CXCR4 (Figure 1b). These results indicate that CXCR4 is expressed on both feline astrocytes and microglia.

The amino acid sequences of human and feline CXCR4 have a high homology (Willett *et al*, 1997), and thus, human SDF-1α and SDF-1β (PeproTech) were used as ligands for CXCR4 in this experiment. In addition, AMD3100 (Schols *et al*, 1997), which has been shown to block HIV-1 entry via the CXCR4, was used as a specific probe for CXCR4 and was kindly provided by G Henson (AnorMED). Monoclonal antibody (mAb) against human CXCR4 (12G5) was purchased from R&D Systems.

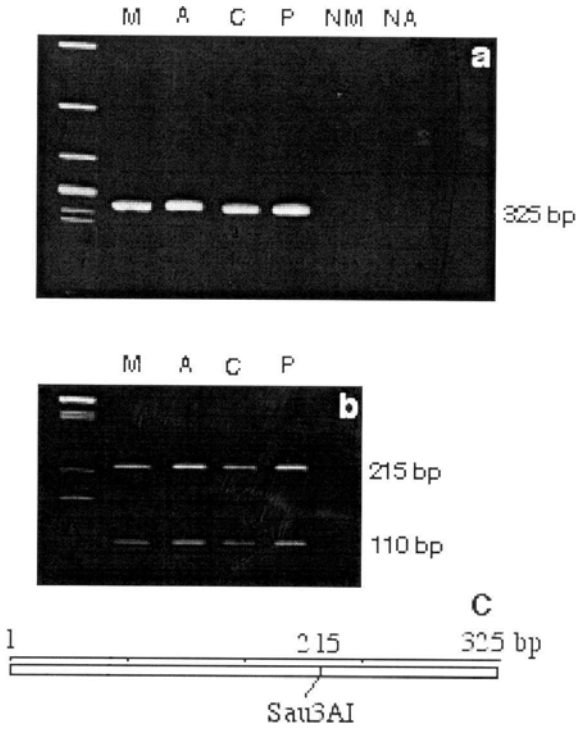

Figure 1 The expression of CXCR4 mRNA in feline cultured cells. a) PCR products using feline CXCR4 primer (325 bp). The cells presented are microglia (M), astrocytes (A), CrFK cells (C), peripheral lymphocytes (P), microglia with no reverse transcriptase added (NM), and astrocytes with no reverse transcriptase added (NA). b) Digested products (215 bp, 110 bp) by restriction enzyme Sau 3AI. The cells presented are microglia (M), astrocytes (A), CrFK cells (C), and peripheral lymphocytes (P). HincII-digested fX174 DNA was used as size marker. c) Restriction map of Sau3AI sites deduced from b).

A blocking assay was conducted using FIV Petaluma strain, which productively infects feline astrocytes. The feline astrocyte-rich cultures were processed as described above. The astrocytes used for the blocking assays were re-seeded from the primary culture at a density of 10^5cells/ml in a 48-well culture plate. The virus was amplified by culturing chronically infected CrFK cells (ATCC). The culture supernatant was clarified by low-speed centrifugation (13,000 rpm, 10 min), and the virus was concentrated by high-speed centrifugation (45,000 rpm, 60 min). An aliquot of the concentrated culture supernatant was stored at −80°C. The virus stock was titrated by measuring the core antigen p24 (FIV p24 ELISA Kit; IDEXX Corp) in the culture medium after infection of astrocytes with 4-fold dilutions of virus.

In the FIV blocking assay, astrocytes were incubated for 30 min at room temperature in the presence of SDF-1α, SDF-1β, AMD 3100, 12G5 at the concentration of 1.0 ng–1.0 mg/ml or a complete medium alone, and then 10 μl of FIV stock solution, which produce minimum but certainly detectable dose of p24 antigen in 100% well, were added for 1 h. After four washes with IMDM, cells were further maintained in the complete medium containing a ligand or mAb at half the concentration used in blocking, or in the complete medium alone. The results 9 days after infection were evaluated based on the time course of p24 expression (Figure 2). As shown in Figure 3, both SDF-1α and SDF-1β dose-dependently inhibited p24 production, and the inhibition of FIV infection reached more than 75% at a dose of 0.1–1 mg/ml. A 50% inhibition by SDF-1α was achieved at a concentration of 20 ng/ml. The effective concentrations of SDF-1α obtained here were similar to the concentrations described in the infections of CrFk cells with FIV (Hosie *et al*, 1998) and of lymphocytes with HIV-1 (Bleul *et al*, 1996). The antiviral activity of AMD3100 was also dose-dependent. It inhibited more than 50%

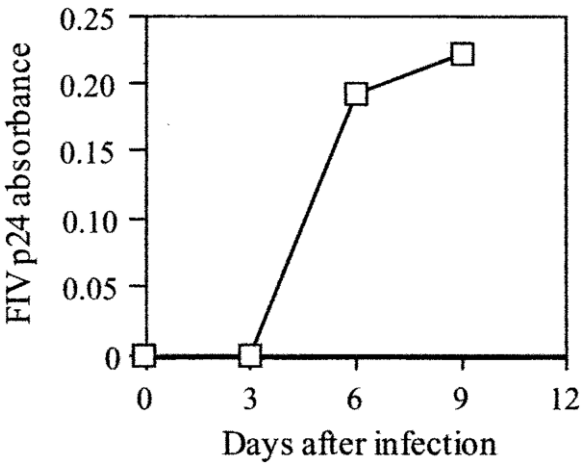

Figure 2 Typical time-course of FIV p24 production in feline primary astrocytes. CrFK-adapted FIV-infected astrocytes started to produce p24 from the 6th day after infection.

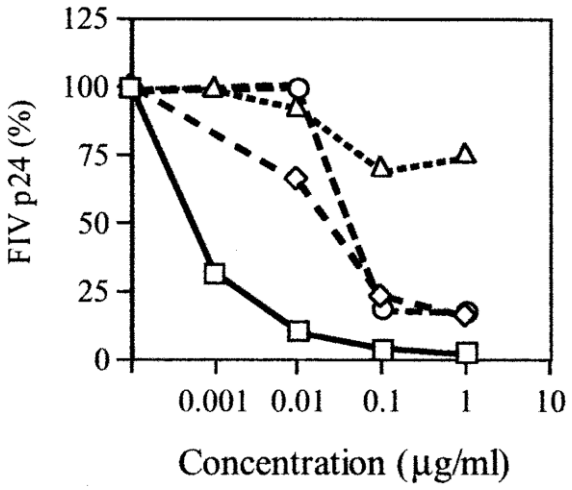

Figure 3 Inhibition of FIV infection of primary astrocytes by CXCR4 ligands. The cells were incubated with SDF-1α (diamond), SDF-1β (circle), AMD3100 (square), or mAb against CXCR4 (triangle) at the concentrations of 1~1000 ng/ml before virus infection. The results 9 days after were evaluated. The data are expressed as the percentage of p24 production with CXCR4 ligands relative to the control without ligand and are mean for triplicate wells.

of p24 production at a dose of 1.0 ng/ml, and completely inhibited at 100 ng/ml. Thus, AMD3100 appeared much more effective than the natural ligands in inhibiting FIV infection in astrocytes. The higher effectiveness of AMD 3100 agrees with the result described in the CrFk cells infection with FIV (Egberink *et al*, 1999; Richardson *et al*, 1999). AMD3100 is a class of antiviral compounds that specifically interact with the CXCR4 chemokine receptor and selectively inhibit the replication of CXCR4-dependent virus, including the T-tropic virus of HIV-1 and HIV-2. This compound has a 50% inhibition at the range of 1–10 ng/ml in those infections (De Clercq *et al*, 1994). Furthermore, it has been confirmed that AMD3100 remains active against the SDF-1α-resistant HIV-1 (Schols *et al*, 1998). In the present study, the effectiveness of AMD 3100 in blocking FIV infection was also shown in feline astrocytes at lower concentrations than for SDF-1α. On the other hand, the 12G5 failed to block the infection of FIV in astrocytes, a finding that was also supported by the observation in CrFK cells by Willett *et al* (1997) and Egberink *et al* (1999). As it was shown that the 12G5 could actually stain feline lymphocytes and neural cells by immunohistochemistry (unpublished data), this failure suggests that 12G5 may utilize the other than the epitope on CXCR4 molecule recognized by FIV to infect astrocytes. The blocking with these ligands of CXCR4 indicates that FIV indeed utilizes CXCR4 to infect astrocytes as well as it does in CrFK cells and lymphocytes.

Although FIV and HIV-1 have been reported to have many common biological and clinical features, it was not until the discovery of CXCR4 that the similarity in the pathogenic mechanisms of these infections was confirmed. Specifically, the second extracellular loop

of CXCR4 was recognized as the major determinant of usage in CXCR4-dependent infection of both FIV (Willett *et al*, 1998) and HIV (Lu *et al*, 1997).

The apoptosis of neurons and astrocytes has been reported to be induced by HIV-1 in brain tissues from AIDS patients (Adie-Biassette *et al*, 1995; Petito and Roberts, 1995), a finding that was later confirmed in *in vitro* systems using human brain mix culture (Shi *et al*, 1996). According to that study, the apoptotic stimuli were induced by the effects of soluble factors produced by HIV-1 infection on microglia/macrophages and were not the result of direct viral infection. This assertion was made on the grounds that HIV-1 is not thought to replicate in neurons and that apoptosis was not induced until 1–2 weeks after viral production. Furthermore, it was demonstrated that blood-derived CXCR4-dependent HIV-1 induced the apoptosis of neurons to a greater degree than brain-derived M-tropic HIV-1 (Ohagen *et al*, 1999). However, it was also reported that, alike gp120 of HIV-1, SDF-1α elicits apoptosis in neural cell lines and that this apoptosis is blocked by antibody against CXCR4 (Hesselgesser *et al*, 1998). The results of that study indicate that CXCR4 contributes to neuronal death caused by HIV-1. Although HIV-1 gp120 and SDF-1 seem to induce apoptosis directly in neuronal cell lines (Hesselgesser *et al*, 1998), apoptosis was not induced until 1–2 weeks after viral production in an experiment using human brain mix culture (Shi *et al*, 1996) including astrocytes, neurons, microglia, and fibroblasts.

Taken together, the mechanisms of ADC in the brain seem complex, probably involving some potentially neurotoxic factors and many regulatory factors. It has been demonstrated that astrocytes may play an important role in the mechanism of neuronal death caused by HIV-1 because astrocytes function as immune regulatory cells and negatively or positively regulate macrophage activation (Notett *et al*, 1995). This is further supported by the fact that SDF-1, which is mainly produced by astrocytes, is upregulated in astrocytes exposed to HIV-1-infected monocytes-derived macrophages-conditioned medium (Zheng *et al*, 1999).

In addition, although HIV-1 has been thought to productively infect microglia/macrophages and non-productively infect astrocytes in CNS, recent increasing studies reveal that astrocytes may be activated to produce virus and serve as a reservoir (Tornatore *et al*, 1991; Gorry *et al*, 1998; Janabi *et al*, 1998). Astrocyte loss caused by virus infection may also result in neuronal death because they support neuronal homeostasis by producing nerve growth factor (Cohen, 1960). Nonetheless the mechanism of astrocytes infection has not yet been clarified. Our results underline the importance of CXCR4 in astrocytes in viral infections. Thus, the blocking of CXCR4 with ligands in astrocytes may constitute an important strategy in AIDS dementia complex therapies.

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