Brief Definitive Report

CXCR4/fusin Is Not a Species-specific Barrier in Murine Cells for HIV-1 Entry

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Summary

Since some murine cells expressing human CD4 fail to internalize HIV-1, another block was thought to be located at the level of viral entry in addition to CD4. Recently, CXCR4 was shown to function as a coreceptor for T cell line-tropic HIV-1 entry. Here we demonstrated that cells expressing murine CXCR4 and human CD4 fused with cells expressing the env proteins derived from T cell line-tropic HIV-1 and were infected with T cell line-tropic HIV-1 strains. In contrast, the same cells were not infected with chimeric clones constructed by substitution of monocyte- or macrophage-tropic strain-derived env region or V3 region into T cell line-tropic HIV-1, indicating V3 loop of envelope protein is required for murine CXCR4-mediated HIV-1 entry. We conclude that murine CXCR4 is not a species specific barrier to the entry of T cell line-tropic HIV-1.

Identifying mechanisms responsible for species specific tropism of HIV-1 may be important to develop an animal model simulating HIV-1 infection as well as to unravel cellular factors for viral infection. Mice are convenient, low cost, well-characterized laboratory animals but are not a natural target of HIV-1, and murine cells present some blocks to viral infection. The first block is located at the level of viral binding to murine cells. Human CD4 binds to HIV-1 but its murine counterpart does not (1). Moreover, previous studies have revealed that when human CD4 is expressed in vitro at the surface of some murine cells including T cell lines, binding of HIV occurs but entry does not (2). This result demonstrated that murine cells expressing human CD4 do not support viral entry and suggested that the defect is due to the absence of another human specific coreceptor required for membrane fusion.

HIV-1 strains exhibit marked differences in their abilities to infect CD4-positive cells. Some strains infect monocytes, classified as monocyte- or macrophage-tropic strains (M-tropic) and other strains infect T cell lines, classified as T cell line tropic (T-tropic). As HIV-1 infection progresses, the initially dominant M-tropic viruses are usually replaced by T-tropic viruses (3, 4, 5). In 1996, CXCR4/fusin, a G protein coupled receptor with seven transmembrane helices, was shown to be essential for entry of T-tropic HIV-1 into human CD4-positive cells (6). The results prompted us to ask whether the function of CXCR4 as a coreceptor of viral entry is species specific. Recently, murine CXCR4 that was shown to be a receptor for CXC chemokine PBSF/SDF-1 (7–9) was isolated and has 90% amino acid identity with human CXCR4 (7).

In this study, we established cells transfected with both human CD4 and murine CXCR4 and examined whether HIV-1 coreceptor CXCR4 is an intrinsic barrier in mouse cells for viral entry.

Materials and Methods

Cell Lines. Mouse NIH 3T3 cells, human small intestine epithelial cell-derived SW 480 cells and human glioma cell lines U 87 M G were cultured in DM EM with 10% FCS. Human HeLaS3 cells were cultured in RPMI1640 with 10% FCS. Human osteosarcoma-derived HOS cells were cultured in Eagle MEM with 1% Nonesential amino acid (GIBCO BRL, Gaithersburg, MD) and 10% FCS. DW 34 cells were provided by S.-I. Nishikawa (Kyoto University, Kyoto, Japan).

Viruses. HIV-1 strain, NL 432 was provided by A. Adachi (University of Tokyo, Japan) (10). IIIB was provided by S. Harada (Kumamoto University, Kumamoto, Japan) (11). SF162 was provided by J.A. Levy (University of California, Los Angeles, Los Angeles, CA) (12).
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San Francisco) (12). HIV-1 chimeric clones, NL432env-162 and NL432v3-162, were provided by Y. Isaka (Shionogi Institute). Recombinant vaccinia viruses, Vac Env (NL432env), Vac Env 162 (SF162 env), and V N T4 (CD4) were provided by T. Shioda (University of Tokyo, Tokyo, Japan). LO-T7 (T7 polymerase) was provided by M. Kohara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan).

Transfection of Cell Lines. NIH3T3 cells were plated overnight in 24-well plates at 5 × 10⁴ cells per well, and transfected with coreceptor in pBPluescript by lipofection. After 4 h, cells were washed with PBS, added by culture medium, incubated at 37°C overnight, and used for fusion assay. NIH3T3 cells and HOS cells were plated overnight in 6-cm dishes at 5 × 10⁶ cells per dish. SW 480 cells were transiently transfected with 5 μg of coreceptor in pEF-BO5 and 7.5 μg of T4-Neo and 2.5 μg of LTR-Eco R (β-Gal)-Neo by modified calcium phosphate method (13). HOS cells, stably expressing human CD4 and LTR-β-Gal were transiently transfected with 15 μg of coreceptor by the same method. Cells were incubated at 35°C in 3% CO₂ overnight, washed with PBS (−), harvested with 0.5 mM EDTA/PBS (-), passed to 12-well plate and incubated at 37°C overnight. Next day, cells were tested for fusion assay.

RT-PCR. Total RNA was isolated and 3.0 μg was used for cDNA synthesis using Ready to Go (Pharmacia, Uppsala, Sweden). One-twentieth of this reaction was used as a template for PCR amplification for 40 cycles at 92°C for 0.5 min, 55°C for 1 min, and 74°C for 2 min. The primer pairs were for murine glyceraldehyde 3-phosphate dehydrogenase (G3PDH; CLONTECH, Palo Alto, CA) for murine CXCR4: 5'-TACGCGGC CGCT TGCCATGGACCGTA-3' and 5'-GCCTGCA ATTTGGCAT AAGGTTAGCTG-3'.

Intracellular Ca²⁺ Response to Murine β-Gal and HIV-1 Env Protein. We used mice (1 × 10⁶ cells/well) in 24-well plate by infection of the recombinant vaccinia viruses. Human CD4, ω subunit of β-gal and T7 RNA polymerase were introduced into target NIH3T3 cells transiently transfected CHO cells loaded with fura-PE3 were done as described (7).

Cell Fusion Assays. To quantify cell–cell fusion events, we used a modified fusion assay based on α-complementation of β-galactosidase (β-gal) (Shida, H., manuscript in preparation). In brief, α subunit of β-gal and env proteins were introduced into effector HeLaS3 cells. Then NIH3T3 cells were transfected with plasmids containing human CXCR4-4 or CCR5 or murine CXCR4. Effector and target cells were mixed together and incubated. If fusion occurred, the cyttoplasmic contents of the fused cells lead to activation of β-gal. As shown in Fig. 1, HeLaS3 cells expressing the env proteins derived from the T-tropic HIV-1 strain NL432 readily fused with NIH 3T3 cells expressing human CXCR4 and human CD4, but not with cells expressing human CCR5 and human CD4. Surprisingly, δt fusion of murine CXCR4 and human CD4. HeLaS3 cells expressing the env proteins derived from the M-tropic strain SF162 fused with cells expressing human CCR5 and human CD4, but not with cells expressing murine CXCR4 in conjunction with human CD4.

Second, we examined whether murine CXCR4 also allows for virus infection to target cells. Since murine cells, including NIH 3T3 that expressed human CXCR4 and CD4 supported HIV-1 replication much less efficiently (data not shown), we used three types of human cells, small intestine epithelial cell-derived SW 480 cells, osteosarcoma-derived HOS cells and glioma cell-derived U87MG as the target cells for viral infection. Cells were transfected with an integrated HIV-1 long terminal repeat (LTR)-driven reporter gene, lacZ. If viruses enter the cells, HIV-1 encoded transactivating protein Tat is expressed and induces the expression of lacZ. In addition, they were transfected with human CD4 plus chemokine receptors. The cells were infected with T-tropic virus strains, NL432 or IIIB, or an M-tropic

Infected Assays. Human SW 480 or HOS cell lines bearing human CD4 and coreceptors were seeded as monolayers in 12-well tissue culture plates. To each well, 200 μl of HIV-1 virus-containing fluid (reverse transcriptase [RT] activity, 2 × 10⁴ RT/ml for SF162, NL432env-162, NL432v3-162, 5 × 10⁴ RT/ml for IIIB, and 3 × 10⁴ RT/ml for NL432) was added, and the plates were incubated for 2 h at 37°C in 5% CO₂ and 2.5 ml of culture medium was added. An aliquot was removed after 4 days of infection and 400 μl of reporter lysis buffer (Promega) was added and frozen to −80°C and thawed. Then samples were transferred to eppendorf tubes, centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was assayed for β-gal activity by Luminescent β-gal Detection Kit (CLONTECH).

Results

First, to determine if murine CXCR4 allows HIV-1 env-mediated membrane fusion, we used the assay system in which fusion between effector env-expressing cells and target coreceptor- and CD4-expressing cells leads to activation of a reporter enzyme. In this assay, effector HeLaS3 cells were infected with a recombinant vaccinia virus that expresses α subunit of β-gal and HIV-1 env protein. We used NIH 3T3 cells as the target cells since no CXCR4 was seen and 1,000 nM PBSF/SDF-1, a ligand for CXCR4 did not induce an increase in intracellular free Ca²⁺ in NIH 3T3 cells (Fig. 1, A and B). The target NIH 3T3 cells were infected with a recombinant vaccinia virus that expresses ω subunit of β-gal, T7 polymerase and human CD4. Then NIH 3T3 cells were transfected with plasmids containing human CXCR4 or CCR5 or murine CXCR4. Effector and target cells were mixed together and incubated. If fusion occurred, the cytoplasmic contents of the fused cells lead to activation of β-gal. As shown in Fig. 1, HeLaS3 cells expressing the env proteins derived from the T-tropic HIV-1 strain NL432 readily fused with NIH 3T3 cells expressing human CXCR4 and human CD4, but not with cells expressing human CCR5 and human CD4. Surprisingly, they also fused with cells expressing murine CXCR4 and human CD4. HeLaS3 cells expressing the env proteins derived from the M-tropic strain SF162 fused with cells expressing human CCR5 and human CD4, but not with cells expressing murine CXCR4 in conjunction with human CD4.
strain SF162 and harvested. As shown in Fig. 2 A, NL432 or IIIB entered SW 480 that expressed both murine CXCR4 and human CD4 equally compared to the cells that expressed both human CXCR4 and human CD4, consistent with the results of the env-mediated fusion assay described above. Entry did not occur when human CCR2b or CCR5 was expressed in place of CXCR4. SF162 entered cells expressing both human CCR5 and CD4 but not cells expressing human or murine CXCR4 plus human CD4. Similar results were obtained from using HOS cells (Fig. 2)

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**Figure 1.** Murine CXCR4 supported membrane fusion mediated by T cell line-tropic HIV-1 env protein. (A) RT-PCR analysis of murine CXCR4 mRNA expression on NIH3T3 cells and DW34 cells. (B) Intracellular Ca²⁺ response of NIH3T3 cells and CHO cells transfected with murine CXCR4 in response to murine PBSF/SDF-1. Rise in intracellular Ca²⁺ concentration is represented by the increase in relative fluorescence. (C) Quantitation of fusion by β-galactosidase activity assay. NIH3T3 target cells were infected with a recombinant vaccinia virus that expresses human CD4, T7 polymerase and α subunit of β-gal. Then the cells were transfected with murine CXCR4 or human CXCR4 or CCR5. HeLaS3 effector cells were infected with a recombinant vaccinia virus that expresses α subunit of β-gal and env proteins derived from HIV-1 strains NL432 or SF162. Cells were allowed to fuse and assayed for β-gal activity.

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**Figure 2.** Murine CXCR4 supported infection of a T cell line-tropic HIV-1 virus. SW480 cells (A), HOS cells (B), or U87MG cells (C) were cotransfected with human CD4 plus chemokine receptors, and then infected with HIV-1 strains NL432, IIIB, or SF162. Cell lysates were assayed for β-gal activity.
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B) or U87MG cells (Fig. 2 C) in place of SW480 cells. Thus, murine CXCR4 supports entry of the T-tropic virus strains into target cells and may not affect the synthesis, integration, and expression of proviral DNA in human cells.

Human CXCR4-mediated HIV-1 entry has been shown to be inhibited by the monoclonal antibody directed against the V3 loop (6). Then, to confirm that the function of murine CXCR4 can replace the function of human CXCR4 further, we investigated whether the V3 loop of envelope gp120 is required for murine CXCR4-mediated HIV-1 entry. SW480 cells expressing human CD4 and chemokine receptors were infected with viral chimeric clones, NL432env-162 and NL432V3-162. As shown in Fig. 3 A, NL432env-162 was constructed by substitution of an M-tropic strain SF162 env region into a T-tropic strain NL432 proviral clone. NL432V3-162 was constructed by substitution of an SF162 V3 region into a NL432 proviral clone. Although NL432 entered SW480 cells expressing murine CXCR4 and human CD4, both NL432env-162 and NL432V3-162 failed to enter those cells. Both NL432env-162 and NL432V3-162 entered SW480 cells expressing human CCR5 and human CD4. These results revealed that the V3 region is required for viral entry supported by murine CXCR4 as well as human CXCR4.

Discussion

This study revealed that murine CXCR4 could support T-tropic HIV-1 env-mediated membrane fusion and viral entry, indicating that there is no species specific barrier at CXCR4. Previous studies showed that when human CD4 is expressed in vitro at the surface of murine lymphoid or non-lymphoid cells including NIH3T3 and T cell clone 3DT, binding of HIV-1 occurs but entry does not (2). One explanation of the results is that the murine cells expressing human CD4 do not express CXCR4 at the cell surface. In fact, CXCR4 mRNA and murine PBSF/SDF-1-responsive CXCR4 were not seen in NIH3T3 cells (Fig. 1, A and B). However, murine CXCR4 is expressed in both double-positive (CD4+CD8+) and single positive (CD4+CD8-, CD4-CD8+) thymocytes (7). It is important to determine whether 3DT cells express CXCR4. Recently, some murine cells that express human CD4 were shown to be infected by T-tropic HIV-1 strains (15), supporting our conclusions. In addition, the previous study showed that a rat cell line can be infected by T-tropic HIV-1 strains (16). This result, together with our results, suggest that rat CXCR4 is also functional for HIV-1 entry.

It has been reported that a murine counterpart of CCR5, an entry coreceptor for M-tropic HIV-1 (17–21) does not support viral entry (22), indicating that species specific limitation to a coreceptor function for M-tropic HIV-1 is distinct from that for T-tropic HIV-1. The difference may be due to high amino acid sequence conservation of CXCR4 between species compared to other chemokine receptors, including CCR5. The amino acid sequence of murine CXCR4 has 90% identity with that of human CXCR4, while CCR5 and CXCR2 are 82 and 71% identical between mouse and human. The strong conservation of CXCR4 is consistent with the unique functions of its ligand, PBSF/SDF-1 among chemokines, including MIP-1α, MIP-1β, and RANTES, ligands of CCR5. PBSF/SDF-1 has essential functions in development including hematopoiesis and cardiogenesis (23) while other chemokines are thought to be involved in trafficking of leukocytes in inflammation.

The previous studies and the result that a murine cell line NIH3T3 transfected with human CD4 and coreceptors sup-
ported HIV-1 entry but produced virus particles much less efficiently than human cells (data not shown) suggest the absence of intracellular molecules that are critical for viral replication in mice (24–26). However, we could develop a murine HIV-1 infection model by constructing the transgenic mice containing the human DNAs of the molecules responsible for species specific barriers. Our results indicate that we do not have to introduce human CXC CR4 into those mice and provide useful information to develop an animal model to simulate all phases of infection, since the physiological expression of CXC CR4 may be involved in initiation and exacerbation of the shift from M-tropic to T-tropic HIV-1 strains leading to advanced clinical disease.

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