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CXCR4-Tropic, But Not CCR5-Tropic, Human Immunodeficiency Virus Infection Is Inhibited by the Lipid Raft-Associated Factors, Acyclic Retinoid Analogs, and Cholera Toxin B Subunit

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Abstract

Development of an effective low-cost anti-acquired immunodeficiency syndrome (AIDS) drugs is needed for treatment of AIDS patients in developing countries. Host cell lipid raft microdomains, which are enriched with cholesterol, glycolipids, ceramide, and gangliosides, are important for human immunodeficiency virus type 1 (HIV-1) entry. Retinoid analogs have been shown to modulate ceramide levels in the cell membrane, while cholera toxin B subunit (CT-B) specifically binds to the ganglioside GM1. In this study, we found that the acyclic retinoid analogs geranylgeranoic acid (GGA) and NIK-333 as well as CT-B efficiently attenuate CXCR4-tropic, but not CCR5-tropic, HIV-1 vector infection. We also found that GGA and NIK-333 suppress CXCR4-tropic HIV-1 infection by attenuating CXCR4 expression. CT-B also attenuated CXCR4-tropic HIV-1 infection, but did not suppress CXCR4 expression. These results suggest a distinct role for lipid raft microdomains in CXCR4- and CCR5-tropic HIV-1 infections and illuminate novel agents for the development of AIDS therapy.

Introduction

HIGHLY ACTIVE ANTIRETROVIRAL therapy (HAART), which suppresses human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, protease, and integrase, has been found to be an effective treatment against acquired immunodeficiency syndrome (AIDS). In fact, many patients infected with HIV-1 do not progress to AIDS in developed countries due to implementation of HAART. However, HIV-1/AIDS continues to be a serious problem, as many HIV-1-infected patients in developing countries do not have access to effective anti-HIV-1 drugs due to the prohibitive cost of the therapy, and thus, the numbers of HIV-1-infected patients are increasing worldwide. In addition, HIV-1 variants resistant to current drugs have appeared.[1] To resolve these problems, novel, low-cost drugs that inhibit HIV-1 infection are critical.

Lipid raft microdomains of target cell membranes are required for HIV-1 infection.[2–6] Lipid rafts are enriched with cholesterol, glycolipids, and ceramide.[7] Extraction of cholesterol from cell membranes,[4,6] binding of cholesterol with various factors,[2,8] and inhibition of biosynthesis of cholesterol[9,10] or glycolipids[11–13] suppress HIV-1 infection, suggesting that cholesterol and glycolipids may be targets for novel anti-HIV-1 drugs. In this study, we examined the effects of lipid raft-associated factors, which were isolated from natural products, on HIV-1 vector infection.

Retinoic acid and its analogs modulate ceramide levels in cell membranes.[14–20] Retinoid analogs may inhibit HIV-1 infection by altering ceramide levels of the target cell membrane. In fact, an all-trans retinoic acid[21] and a weak nuclear retinoid receptor agonist, *N*-(4-hydroxyphenyl) retinamide (4-HPR),[22] inhibit HIV-1 infection[19,23]; however, because 4-HPR has severe toxicities, such as induction of vitamin A deficiency

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symptoms, clinical application of 4-HPR is restricted.[24] Geranylgeranoic acid (GGA), which is a natural acyclic retinoid analog present in medicinal herbs,[25] serves as a weak agonist for retinoid receptors, similar to 4-HPR.[26, 27] NIK-333, which is an artificial acyclic retinoid analog with a structure similar to GGA (Fig. 1), prevents recurrence of hepatocellular carcinoma following oral administration without any obvious side effects in clinical studies of liver cancer patients.[28,29] We analyzed the effects of the acyclic retinoid analogs GGA and NIK-333 on HIV-1 vector infection.

Cholesterol is enriched in lipid raft microdomains and requires their structural maintenance. Extraction of cholesterol from cell membranes by methyl-β-cyclodextrin (MβCD),[4, 6] inhibition of cholesterol synthesis by statin,[9,10] or binding of amphotericin B methyl ester to cholesterol[8] suppresses HIV-1 infection. Plant sterols are cholesterol analogs that reduce serum cholesterol levels by replacing cholesterol.[30] Therefore, plant sterols may function as anti-HIV-1 agents.

Because cholera toxin B subunit (CT-B) specifically binds to the ganglioside GM1, this subunit is frequently used as a lipid raft marker.[4,6] The cytopathic determinant of cholera toxin is subunit A, which has the poly(ADP) ribosylation activity of G-proteins.[31] In contrast, the B subunit has no cytopathic effect. GM1 is enriched in raft microdomains and has been reported to bind HIV-1 envelope (Env) glycoprotein.[32] Additionally, CD4-positive lymphocytes that have elevated levels of another gangliosides, GM3, are highly susceptible to HIV-1 fusion and entry.[11] Therefore, CT-B may inhibit HIV-1 infection without cytopathic effects.

In this study, we examined the effects of these raft-associated factors on HIV-1 vector infection. Our results showed that acyclic retinoid analogs and CT-B efficiently suppressed CXCR4-tropic HIV-1 vector infection, providing novel strategies for the development of CT-B or acyclic retinoid analog treatment for AIDS patients. In contrast, these factors did not affect CCR5-tropic HIV-1 vector infection, suggesting that raft microdomains are involved differently in CXCR4- and CCR5-tropic HIV-1 infections.

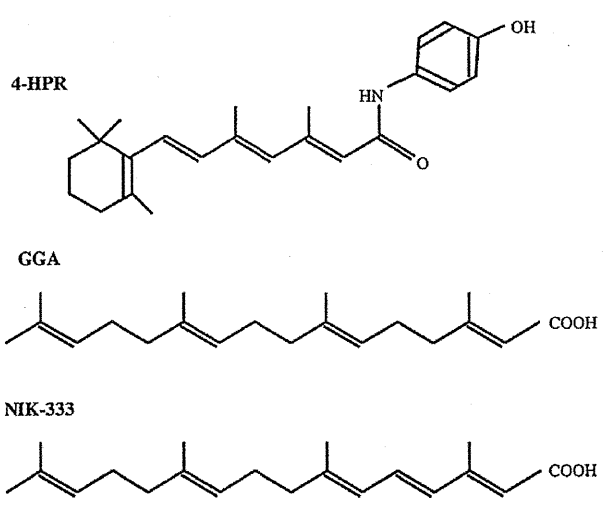

FIG. 1. Chemical structures of 4-HPR, GGA, and NIK-333.

Materials and Methods

Cells

COS7, 293T, NP2, TE671, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) (Wako) supplemented with 8% fetal bovine serum (Biosource) at 37°C in 5% CO_2. NP2 cells expressing CD4 and CXCR4 (NP2/CD4/X4) or CD4 and CCR5 (NP2/CD4/R5) were kindly provided by Dr. H. Hoshino.[33] NP2 cells expressing CD4 and C-terminally HA-tagged CXCR4 (NP2/CD4/X4-HA) were constructed as previously reported.[34] TE671, HeLa, and 293T cells expressing CD4 (TE671/CD4, HeLa/CD4, and 293T/CD4) were constructed with a CD4-encoding murine leukemia virus (MLV) vector as previously reported.[35] MAGIC5 cells, which are derived from HeLa cells, express CD4 and CCR5 and contain the β-galactosidase (β-Gal) gene under control of the HIV-1 long terminal repeat.[36]

Expression plasmids

CXCR4-tropic HXB2 and CCR5-tropic JRFL HIV-1 Env expression plasmids were kindly provided by Dr. Y. Yokomaku (National Hospital Organization Nagoya Medical Center). A VSV-G expression plasmid and expression plasmids required for LacZ reporter gene-containing HIV-1 vector construction were obtained from Invitrogen. An expression plasmid encoding C-terminally HA-tagged CXCR4 was constructed as already reported.[34]

Transduction assay

To obtain HIV-1 vector particles, COS7 cells were transfected with the HIV-1 vector construction plasmids using Fugene transfection reagent (Roche). The transfected cells were washed with D-MEM medium 24 h after transfection and maintained in fresh medium for 24 h. Target cells were either left untreated or pretreated with the retinoid analogs 4-HPR (Sigma-Aldrich), GGA, or NIK-333 for 2 days or with CT-B (Sigma-Aldrich) or stigmasterol (Sigma-Aldrich) for 1 day. GGA and NIK-333 were synthesized by Kowa Company, Ltd. (Tokyo, Japan). The cells were inoculated with culture supernatants from the transfected COS7 cells and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Nacalai) 2 days after inoculation. Blue cells were counted to estimate transduction titer. Approximately 10^4, 10^4, and 10^6 infected cells were detected among cells inoculated by the HXB2 Env-, JRFL Env-, and VSV-G-containing vectors, respectively. To normalize transduction titers, the VSV-G vector was diluted 100 times with medium.

Flow cytometry

To analyze cell surface CD4 expression, suspended cells were either left untreated or treated with an anti-CD4 antibody conjugated with FITC (Sigma-Aldrich). Cell surface expression of CXCR4 or CCR5 was analyzed in suspended cells treated with rat anti-CXCR4 (A80) or anti-CCR5 (T312) monoclonal antibody.[37] As a control, cells were treated with a rat serum. The cells were then washed three times with phosphate-buffered saline (PBS) and treated with an FITC-conjugated anti-rat IgG antibody (Sigma-Aldrich). The stained cells were quantified using a flow cytometer (BD Biosciences).

Western immunoblotting

NP2/CD4/X4-HA cells were treated with the retinoid analogs, and cell lysates were prepared. The cell lysates were subjected to SDS polyacrylamide gel electrophoresis (Bio-Rad) and transferred onto a PVDF membrane (Millipore). The membrane was treated with a mouse anti-HA monoclonal antibody (Covance), and then with an HRP-conjugated anti-mouse IgG antibody (Bio-Rad).

Vector particle binding to target cells

Target cells were incubated with culture supernatants from the HIV-1 vector-producing cells for 1 h at 4°C. The cells were washed three times with PBS, and cell lysates were prepared. HIV-1 Gag p24 levels were measured with a p24 enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix) to estimate the numbers of HIV-1 vector particles bound to the target cells.

Cell fusion assay

The 293T cells were transfected with the HXB2 Env expression plasmid, which also encodes the Tat protein. As a control, 293T cells were transfected with a Tat expression plasmid. The transfected cells were cultured with MAGIC5 cells 24 h after transfection, and cell lysates were prepared from the cells 24 h after the mixed culture. Upon cell fusion, the Tat protein induced β-Gal expression. β-Gal activity in the cell lysates was measured to estimate cell fusion capability.

Statistical analysis

Differences between two groups were determined by the Student's t-test. The difference was considered statistically significant if the p-value was < 0.05 for all tests.

Results

Acyclic retinoid analogs and CT-B inhibit CXCR4-tropic HIV-1 vector infection

To assess whether retinoid analogs inhibit HIV-1 vector infection, target cells were pretreated with 4-HPR, GGA, or NIK-333 for 2 days. The chemical structures of the analogs are shown in Fig. 1. NP2 cells expressing CD4 and CXCR4 (NP2/CD4/X4), NP2 cells expressing CD4 and CCR5 (NP2/CD4/R5),[33] and HeLa cells expressing CD4 (HeLa/CD4)[35] were used as target cells. All of the retinoid analogs inhibited infection by a CXCR4-tropic HXB2 Env-carrying HIV-1 vector (Fig. 2A). Previous reports indicated that 4-HPR inhibits HIV-1 infection,[23] and this result is consistent with our findings. In addition, cell viability was not affected by the analog treatment under these conditions. These results indicate that the acyclic retinoid analogs GGA and NIK-333 as well as 4-HPR inhibit CXCR4-tropic HIV-1 infection.

VSV-G-mediated infection is independent of lipid rafts,[4,6] so we assessed whether VSV-G-pseudotyped HIV-1 vector infection is also attenuated by the retinoid analogs. VSV-G-pseudotyped HIV-1 vector infection was not significantly affected by the retinoid analogs (Fig. 2B). Similarly, infection by HIV-1 vector pseudotyped with the Env protein of the CCR5-tropic JRFL strain was not inhibited by the retinoid analogs (Fig. 2C). These results indicate that the retinoid analogs specifically suppress CXCR4-tropic HIV-1 Env-mediated infection but not VSV-G- and CCR5-tropic HIV-1 Env-mediated infection and that the retinoid analogs inhibit CXCR4-tropic HIV-1 infection by a mechanism other than a cytopathic effect.

We next assessed whether CT-B inhibits HIV-1 vector infection. CD4-expressing TE671 (TE671/CD4), HeLa/CD4, NP2/CD4/X4, and NP2/CD4/R5 cells were pretreated with CT-B for 24 h and then inoculated with HXB2 Env- or JRFL Env-bearing HIV-1 vector in the absence of CT-B. CT-B significantly attenuated CXCR4-tropic Env-mediated infection but not VSV-G-pseudotyped HIV-1 vector infection in TE671/CD4 (Fig. 3A), HeLa/CD4 (Fig. 3B), and NP2/CD4/X4 cells (Fig. 3C). However, CT-B did not inhibit CCR5-tropic Env-mediated infection in NP2/CD4/R5 cells (Fig. 3C). If CT-B inhibited cell growth, this toxin should also suppress VSV or CCR5-tropic vector infection; however, CT-B did not affect cell growth as analyzed by microscopic observation. These results indicate that CT-B specifically suppresses CXCR4-tropic HIV-1 infection by a mechanism other than cell growth inhibition.

Additionally, we assessed whether a plant sterol, stigmasterol, inhibits HIV-1 vector infection. The target cells were pretreated with stigmasterol (80 μg/ml) for 24 h. The transduction efficiency of the HIV-1 vector was not affected by the treatment (data not shown).

Retinoid analogs inhibit CXCR4 cell surface expression

As the acyclic retinoid analogs inhibited CXCR4-tropic HIV-1 vector infection, we next assessed whether these retinoid analogs suppressed cell surface expression of the HIV-1 infection receptors, CD4, CXCR4, and CCR5. 4-HPR did not affect CD4 cell surface expression in HeLa/CD4 cells (Fig. 4A). GGA and NIK-333 treatment elevated CD4 expression, though the acyclic retinoid analogs inhibited CXCR4-tropic HIV-1 vector infection. In contrast, all of these retinoid analogs reduced cell surface CXCR4 expression. Similar results were observed in NP2/CD4/X4 cells, in which CXCR4 is artificially expressed (data not shown). Furthermore, these retinoid analogs did not affect CCR5 expression (Fig. 4B). These results suggest that the retinoid analogs inhibit CXCR4-tropic HIV-1 infection by suppressing CXCR4 cell surface expression.

When NP2 cells expressing C-terminally HA-tagged CXCR4 were treated with the retinoid analogs, expression levels of the HA-tagged CXCR4 were not altered, analyzed by Western immunoblotting using an anti-HA antibody (Fig. 4C). This result suggests that the retinoid analogs inhibit the trafficking of CXCR4 to the cell surface, but do not inhibit CXCR4 expression.

CT-B also inhibited CXCR4-tropic HIV-1 vector infection but not CCR5-tropic HIV-1 vector infection; however, CT-B did not affect cell surface expression of CCR5 (Fig. 4B), CXCR4, or CD4 (Fig. 4C). These results indicate that CT-B inhibits CXCR4-tropic infection by a mechanism other than suppression of CXCR4 expression.

Retinoid analogs and CT-B do not affect HIV-1 particle binding to host cells

We analyzed the effects of the retinoid analogs and CT-B on CXCR4-tropic HIV-1 vector particle binding to the target cells by p24 ELISA. The amount of p24 protein

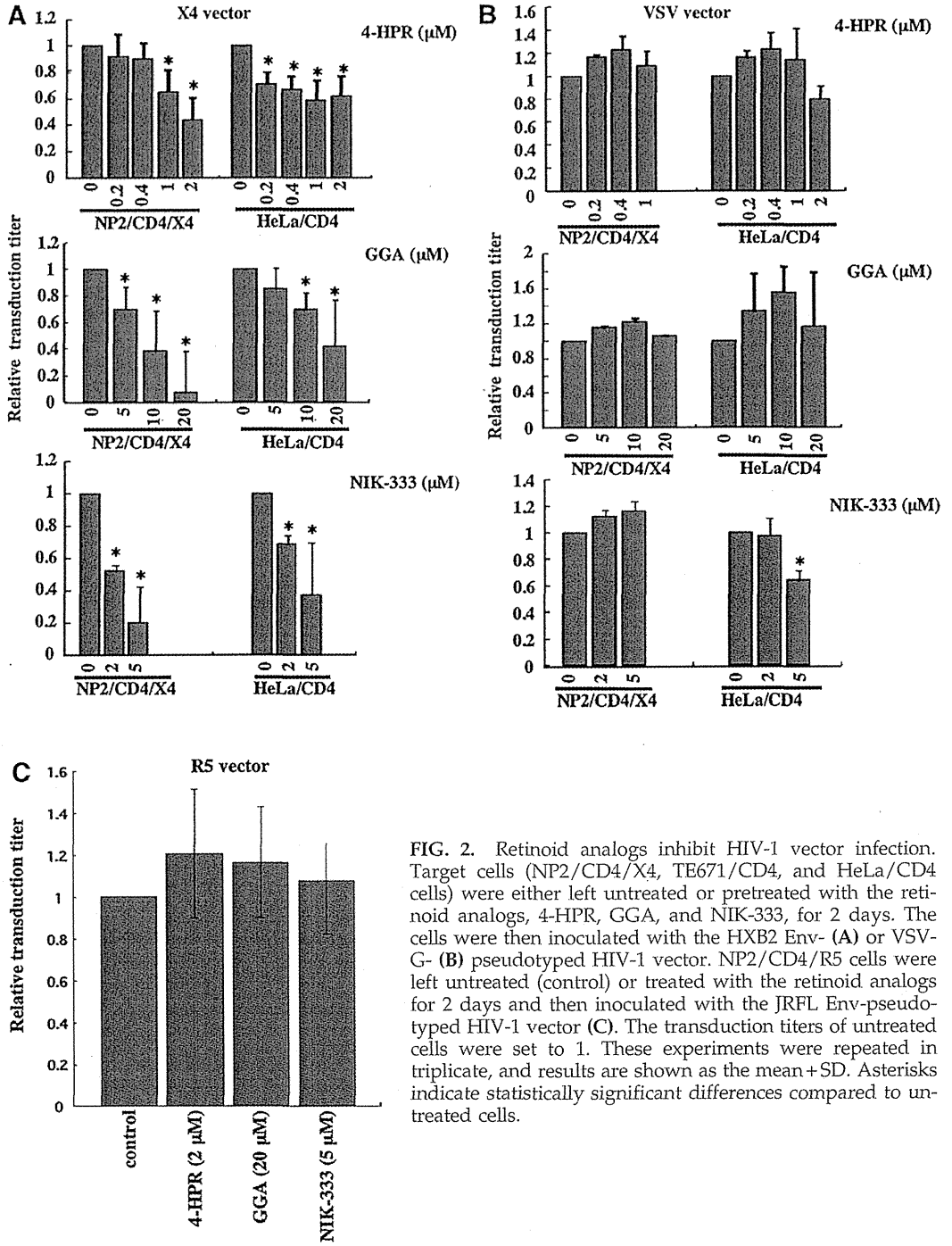

FIG. 2. Retinoid analogs inhibit HIV-1 vector infection. Target cells (NP2/CD4/X4, TE671/CD4, and HeLa/CD4 cells) were either left untreated or pretreated with the retinoid analogs, 4-HPR, GGA, and NIK-333, for 2 days. The cells were then inoculated with the HXB2 Env- **(A)** or VSV-G- **(B)** pseudotyped HIV-1 vector. NP2/CD4/R5 cells were left untreated (control) or treated with the retinoid analogs for 2 days and then inoculated with the JRFL Env-pseudotyped HIV-1 vector **(C)**. The transduction titers of untreated cells were set to 1. These experiments were repeated in triplicate, and results are shown as the mean+SD. Asterisks indicate statistically significant differences compared to untreated cells.

bound to CD4-expressing HeLa cells was higher than that bound to CD4-negative HeLa cells, indicating that vector particle binding is CD4-dependent (Fig. 5A). None of the retinoid analogs (Fig. 5B) or CT-B (Fig. 5C) affected HIV-1 vector particle binding to the CD4-expressing target cells. These results show that the retinoid analogs and CT-B inhibit CXCR4-tropic HIV-1 infection by a mechanism other than suppression of CD4-dependent virion binding to target cells.

Retinoid analogs and CT-B inhibit membrane fusion activity of HIV-1 Env protein

To assess whether the retinoid analogs or CT-B inhibit HIV-1 Env-mediated membrane fusion activity, we analyzed the effects of these agents on HIV-1 Env-induced syncytium formation. HEK293T cells transfected with the plasmid encoding the HIV-1 HXB2 Env and Tat proteins were cocultured with MAGIC5 cells for 24 h, and β-galactosidase activity was

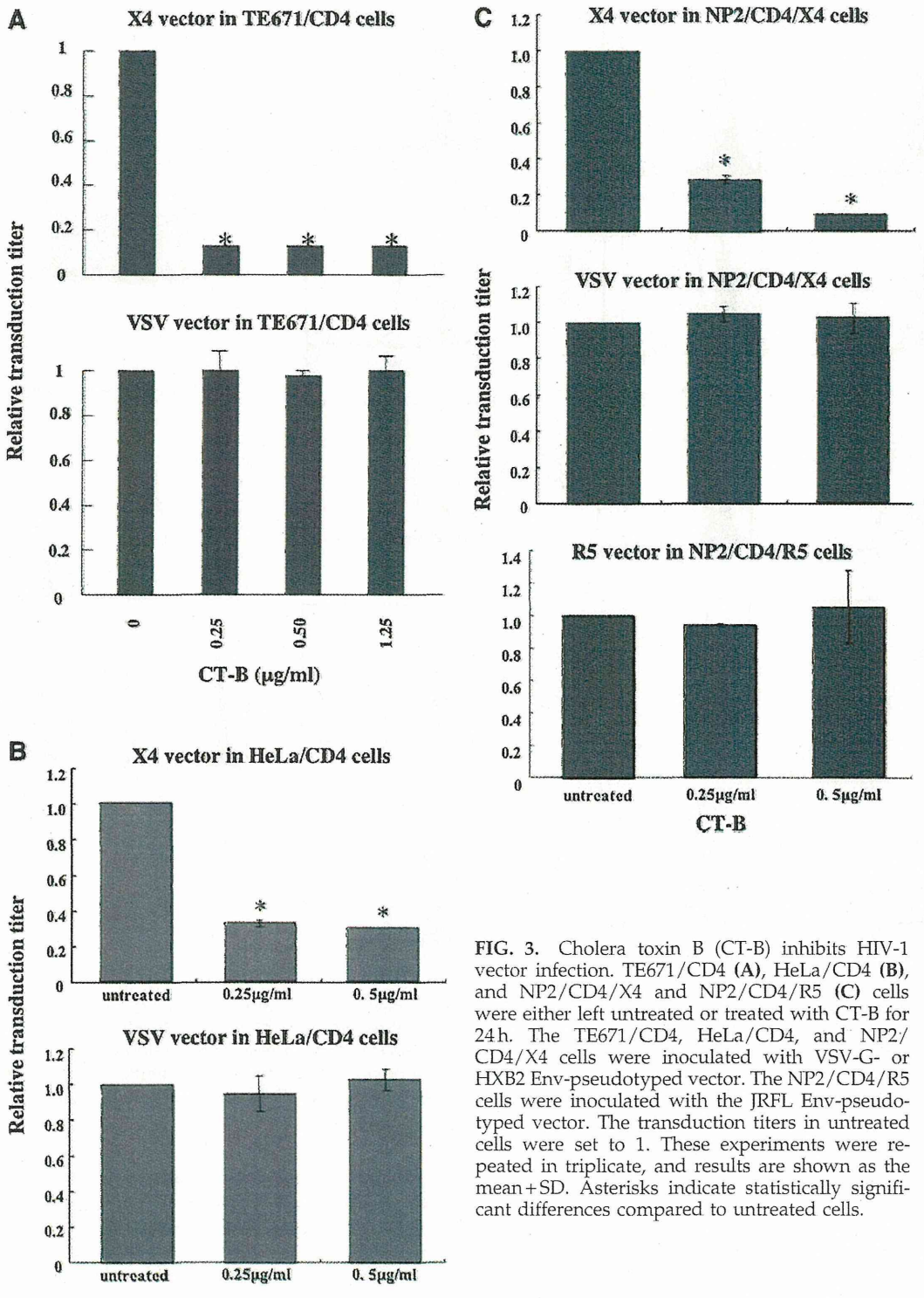

FIG. 3. Cholera toxin B (CT-B) inhibits HIV-1 vector infection. TE671/CD4 **(A)**, HeLa/CD4 **(B)**, and NP2/CD4/X4 and NP2/CD4/R5 **(C)** cells were either left untreated or treated with CT-B for 24 h. The TE671/CD4, HeLa/CD4, and NP2/CD4/X4 cells were inoculated with VSV-G- or HXB2 Env-pseudotyped vector. The NP2/CD4/R5 cells were inoculated with the JRFL Env-pseudotyped vector. The transduction titers in untreated cells were set to 1. These experiments were repeated in triplicate, and results are shown as the mean+SD. Asterisks indicate statistically significant differences compared to untreated cells.

measured in the cell lysates. The retinoid analogs (Fig. 6A) and CT-B (Fig. 6B) suppressed syncytium formation. Direct inhibition of the HIV-1 Env-mediated membrane fusion reaction by these factors would suppress both CXCR4- and CCR5-tropic HIV-1 infections; however, the factors did not affect CCR5-tropic HIV-1 infection (Fig. 2C). Taken together, these results support the hypothesis that retinoid analogs inhibit CXCR4-tropic HIV-1 infection by attenuating CXCR4 expression, although CT-B may affect the HIV-1 entry process between vector particle binding to target cells and membrane fusion.

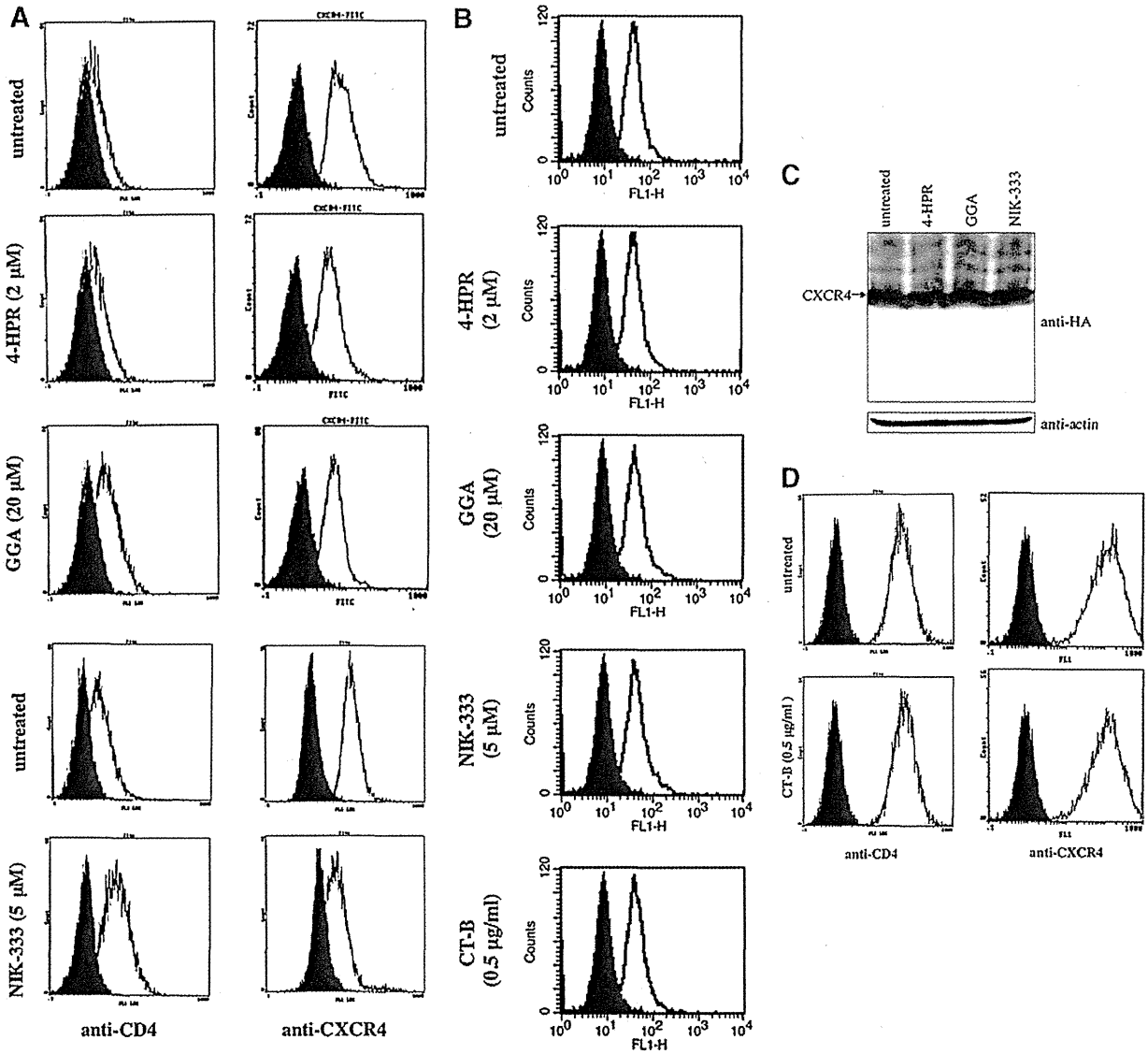

FIG. 4. Retinoid analogs inhibit cell surface expression of CXCR4. Cell surface expression of CD4 and CXCR4 in retinoid analog-treated HeLa/CD4 cells **(A)**, CCR5 in retinoid analog- or CT-B-treated NP2/CD4/R5 cells **(B)**, and CD4 and CXCR4 in CT-B-treated TE671/CD4 cells **(D)** were analyzed by flow cytometry. Closed and open areas indicate untreated cells stained with control serum or with anti-CD4, -CXCR4, or -CCR5 antibody, respectively. Representative results are shown. Expression of C-terminally HA-tagged CXCR4 was analyzed by Western immunoblotting using an anti-HA antibody **(C)**. As a control, actin expression was also analyzed.

Discussion

HAART has dramatically reduced the mortality and morbidity of HIV-1-infected patients in developed countries. However, due to the high cost of HAART, this therapy is limited in developing countries. In addition, HIV-1 variants that are resistant to HAART have emerged. Therefore, development of novel low-cost drugs that inhibit HIV-1 replication is essential.

In this study, we found that the acyclic retinoid analogs, GGA and NIK-333, suppress CXCR4-tropic HIV-1 vector infection similarly to 4-HPR.[23] Additionally, retinoids repress expression of the HIV-1 promoter,[38–40] suggesting that retinoid analogs are possible candidates for a novel anti-HIV-1 therapy. Many reports indicate that vitamin A (retinol) supplementation reduces the mortality of HIV-1-infected patients.[41–44] NIK333, a synthetic acyclic retinoid, is orally effective against liver cancer without severe side effects.[28] GGA also suppresses HIV-1 vector infection and is present in medicinal herbs. These results suggest that oral intake of a natural acyclic retinoid analog may be novel low-cost therapy against AIDS.

We also found that CT-B efficiently suppresses CXCR4-tropic HIV-1 vector infection. Gauthier and Tremblay have shown that CT-B does not inhibit HIV-1 infection, although the concentration of CT-B (10 ng/ml) used in their study was

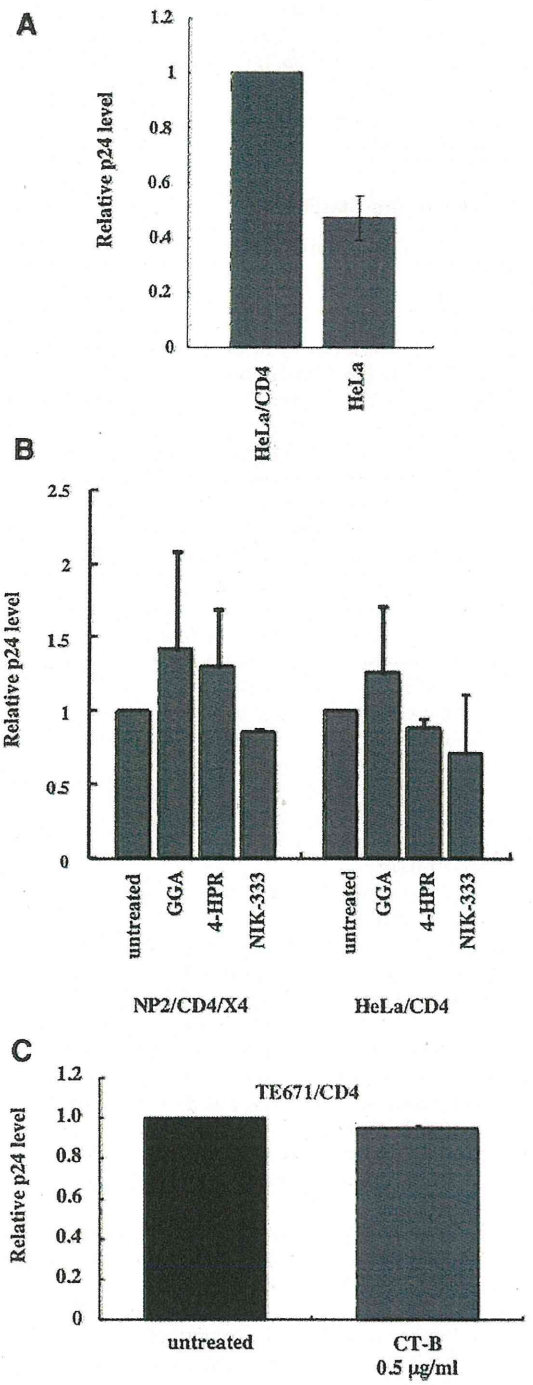

FIG. 5. Retinoid analogs and CT-B do not affect HIV-1 vector particle binding to target cells. HeLa/CD4 and HeLa cells were incubated with the HXB2 Env-containing HIV-1 vector particles at 4°C for 1 h and then washed with phosphate buffered saline (PBS) **(A)**. HIV-1 particles bound to the target cells were measured by p24 ELISA. The p24 levels in untreated HeLa/CD4 cells were set to 1. HIV-1 vector particles bound to the retinoid analog-treated NP2/CD4/X4 or HeLa/CD4 cells **(B)** or to CT-B-treated TE671/CD4 cells **(C)** were measured. The p24 levels in untreated cells were set to 1. These experiments were repeated in triplicate, and results are shown as the mean+SD.

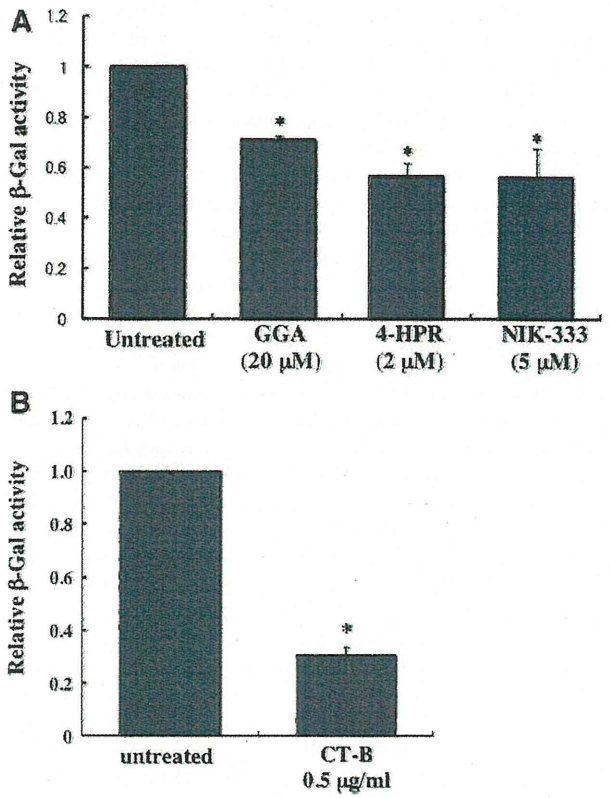

FIG. 6. Retinoid analogs and CT-B inhibit CXCR4-tropic HIV-1 Env-induced syncytium formation. Cell fusion activity of the HXB2 HIV-1 Env protein was measured in untreated and retinoid analog- **(A)** or CT-B-treated **(B)** cells (see Materials and Methods). The β-Gal activities in untreated cells were set to 1. These experiments were repeated in triplicate, and results are shown as the mean+SD. Asterisks indicate statistically significant differences compared to untreated cells.

too low to inhibit HIV-1 infection.[31] Similar to our results with CT-B, pertussis toxin B subunit also inhibits HIV-1 infection.[45–47] Although the receptor for pertussis toxin B oligomer has not yet been identified, the receptor appears to belong to a class of sialylated glycoproteins, with likely candidates being a 43-kDa protein[48] and CD11b/CD18 integrin.[49] Because the CT-B receptor GM1 is not the pertussis toxin B subunit receptor, the mechanisms by which these bacterial toxin B subunits inhibit HIV-1 infection appear to be different.

One route of HIV-1 transmission is through anal sex. As such, if gut bacteria that secrete nontoxic CT-B are present in the rectum, HIV-1 transmission through this route may be suppressed. Gut bacteria genetically engineered to express CT-B may be a useful novel low-cost strategy to prevent HIV-1 transmission through anal sex.

Use of these factors *in vivo*, however, should be approached cautiously. First, our study suggests that the acyclic retinoid analogs modulate cell surface expression of CD4 and CXCR4. Second, CT-B is used as an adjuvant for vaccination.[50,51] Therefore, these agents may induce unexpected effects *in vivo* via activation or perturbation of the human immune system. Further study is required to address this issue.

Other retinoid analogs have been reported to reduce cell surface expression of CXCR4,[52,53] similar to the retinoid analogs

used in this study. This down-regulation of CXCR4 expression is one of the mechanisms by which retinoid analogs inhibit CXCR4-tropic HIV-1 infection. HIV-1 infection is suppressed and influenza virus infection is elevated by 4-HPR through activation of endocytosis[23] without suppression of CXCR4 expression. In this study, VSV-G-mediated infection, which occurs via the endosomes, was not affected by the retinoid analogs. Further study is needed to understand the mechanism of HIV-1 infection inhibition by the retinoid analogs.

The retinoid analogs inhibited CXCR4 expression, while CT-B did not, suggesting that the mechanism of HIV-1 infection inhibition by CT-B differs from that by the retinoid analogs. Interestingly, CT-B inhibited CXCR4-tropic HIV-1 infection but not CCR5-tropic infection. Thus, CT-B may inhibit CXCR4-tropic HIV-1 entry at some point between virion binding to host cells and membrane fusion. CD4 and CCR5, but not CXCR4,[4–6] localize to lipid raft microdomains and constitutively interact.[54,55] It has been reported that CCR5-tropic HIV-1 infection is not dependent upon raft localization of CD4 and CCR5.[56] These results, together with our findings, suggest that CT-B inhibits the HIV-1 Env-induced interaction of CD4 and CXCR4 in lipid rafts and that raft microdomains are differentially involved in CXCR4- and CCR5-tropic HIV-1 infections. CT-B may have no effect on CCR5-tropic HIV-1 infection, because CD4 and CCR5 constitutively interact without binding HIV-1 Env. Recruitment of CXCR4 to CD4-containing raft microdomains by HIV-1 Env, however, has been observed in studies using CT-B as the raft marker.[5,6] Further study is required to understand the mechanism by which CT-B inhibits HIV-1 infection.

The plant sterol stigmasterol did not suppress HIV-1 vector infection. Our group previously reported that MβCD inhibits HIV-1 vector infection and that the addition of cholesterol to the MβCD-treated cells at 50 μg/ml for 30 min recovers infection, suggesting that cholesterol is incorporated into the cell membrane by the addition of cholesterol.[4] Therefore, treatment of cells with stigmasterol at 80 μg/ml for 24 h likely induces uptake of the plant sterol to the cell membrane. These results indicate that this plant sterol does not affect HIV-1 infection. Similar to mammalian cells, plant cells also have lipid raft microdomains in their membranes,[57] and these raft domains are enriched with plant sterols. Therefore, even upon replacement of cholesterol with stigmasterol in mammalian cells, the lipid raft structure is maintained, and HIV-1 infection remains unaffected.

In summary, the acyclic retinoid analogs, GGA and NIK-333, as well as CT-B, efficiently suppress HIV-1 vector infection. Another retinoid analog, 4-HPR, inhibits HIV-1 infection[23] but induces a vitamin A-deficiency syndrome. In contrast, NIK-333 induces no clinical side effects in patients with liver cancer.[28,29] This study suggests that NIK-333 can be used as a novel anti-HIV-1 agent without severe side effects. Additionally, CT-B inhibits CXCR4-tropic, but not CCR5-tropic.

HIV-1 infection, suggesting that host cell lipid raft microdomains are differentially involved in CXCR4- and CCR5-tropic HIV-1 infections.

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Author Disclosure Statement

No competing financial interests exist.

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Review Article

Retrovirus Entry by Endocytosis and Cathepsin Proteases

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Retroviruses include infectious agents inducing severe diseases in humans and animals. In addition, retroviruses are widely used as tools to transfer genes of interest to target cells. Understanding the entry mechanism of retroviruses contributes to developments of novel therapeutic approaches against retrovirus-induced diseases and efficient exploitation of retroviral vectors. Entry of enveloped viruses into host cell cytoplasm is achieved by fusion between the viral envelope and host cell membranes at either the cell surface or intracellular vesicles. Many animal retroviruses enter host cells through endosomes and require endosome acidification. Ecotropic murine leukemia virus entry requires cathepsin proteases activated by the endosome acidification. CD4-dependent human immunodeficiency virus (HIV) infection is thought to occur via endosomes, but endosome acidification is not necessary for the entry whereas entry of CD4-independent HIVs, which are thought to be prototypes of CD4-dependent viruses, is low pH dependent. There are several controversial results on the retroviral entry pathways. Because endocytosis and endosome acidification are complicatedly controlled by cellular mechanisms, the retrovirus entry pathways may be different in different cell lines.

1. Introduction

Retroviruses include many pathogenic agents in humans and animals. Human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV) induce acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia (ATL), respectively. Murine leukemia viruses (MLVs) are also well-studied among retroviruses because the MLVs are used comparatively as animal models of several human diseases (leukemia, immunodeficiency, and neuropathogenic diseases) and as gene transfer tools. In addition, there are animal retroviruses that are important problems in the livestock industry, such as Visna, equine infectious anemia virus, bovine leukemia virus, and Jaagsiekte sheep retrovirus.

Retroviruses contain envelope membranes consisting of lipid bilayers derived from virus-producing cells. Genomes of simple retroviruses such as MLVs encode three essential elements, gag, pol, and env genes. Complex retroviruses including HIV additionally encode accessory genes whose products regulate the retroviral expression and suppress host antivirus factors [1]. The gag and pol genes encode viral structural proteins and enzymes, respectively. These proteins are synthesized as precursor polyproteins and then are cleaved to mature peptides by a protease encoded by the retroviral pol gene.

Retroviral envelope (Env) glycoprotein encoded by the env gene is also synthesized as a precursor protein and is cleaved to surface (SU) and transmembrane (TM) subunits by a cellular protease [2]. Retroviruses enter host cells by fusion between viral envelope and host cell membrane, following the recognition of cognate cell surface receptors. The SU protein binds to the cell surface receptor protein. The TM protein anchors the SU protein to the surface of viral particles and virus-producing cells by the complex formation of SU and TM. The TM protein mediates the membrane fusion reaction. The entry mechanisms of retroviruses are vigorously studied but are not completely understood. Elucidation of the retrovirus entry machinery

would contribute to the development of new therapeutic approaches for retrovirus-induced diseases.

2. Membrane Fusion by Retroviral Env Glycoprotein

Mechanism of membrane fusion by the retroviral TM proteins is described elsewhere in details [3–7] and is similar to those used by envelope proteins of other enveloped viruses [8, 9]. Briefly, the retroviral entry mechanism is proposed as follows. The TM protein is thought to have hairpin-like structure (Figure 1). The binding of SU with its cognate cell surface receptor induces conformational changes of the TM subunit. The N-terminal hydrophobic domain of the TM subunit called fusion peptide is exposed by the conformational change and inserted into host cell membrane. The TM protein then coverts to a trimer-of-hairpins conformation, and viral envelope and host cell membranes approach and mix. Finally, the fusion pore is formed and expanded to derive the viral core into host cell cytoplasm. This conformational change pathway of the TM protein induces the membrane fusion for the retroviral entry into host cells.

3. Retrovirus Receptors

In this section, we will mainly focus on the infection receptors for MLV and HIV, with which entry mechanisms are most extensively studied among retroviruses. Other reviews should be referred to concerning the infection receptors of animal retroviruses in general [10, 11]. MLVs are divided into four groups according to their host ranges and infection interference, and the four groups recognize different cell surface receptors. Ecotropic MLVs infect mouse and rat and bind to cationic amino acid transporter 1 (CAT1) as the infection receptor [12]. Amphotropic MLVs infect many types of mammals, and inorganic phosphate symporter 2 (Pit2) is the amphotropic infection receptor [13, 14]. Polytropic MLVs has a similar host range to the amphotropic MLVs. The amphotropic MLVs cannot infect amphotropic virus-infected cells, because Pit2 are already occupied by the amphotropic Env proteins, called infection interference. Whereas the polytropic MLVs can infect amphotropic virus-infected cells, indicating that the polytropic virus receptor is different from the amphotropic receptor. Polytropic MLVs recognize XPR1 for the infection [15–17], whose physiological function is unknown yet. Xenotropic MLVs recognize the XPR1 as polytropic MLVs, but do not infect mouse cells. These MLV infection receptors are all multimembrane spanning proteins.

The infection receptors of HIV are CD4 and one of chemokine receptors (CXCR4 or CCR5) [18]. However, HIV variants that do not require CD4 for the infection are sometimes isolated from AIDS patients [19, 20] though the infectivity of CD4-independent variants is much lower than that of CD4-dependent viruses [21]. Such CD4-independent HIV variants recognize multimembrane spanning CXCR4 or CCR5 as the sole infection receptor, like the MLVs.

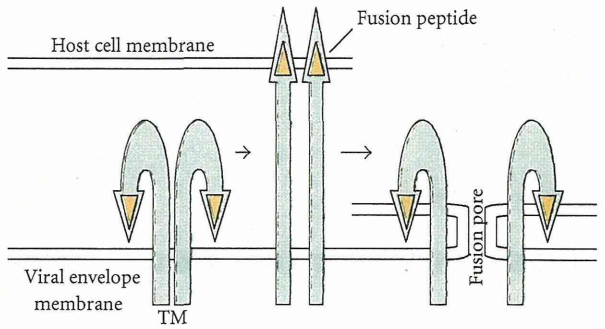

FIGURE 1: Conformational change of retroviral TM subunit for membrane fusion.

CD4 is a single-membrane spanning protein, and HIV variants recognizing CD4 as the sole infection receptor have not been isolated. CD4-independent variants of simian immunodeficiency virus (SIV) are more frequently isolated than CD4-independent HIV variants [22, 23]. It is thought that CD4-independent HIV variants are prototypes of CD4-dependent HIVs [22–24].

4. C-Terminal Tail of Retroviral Env Protein Inhibits Membrane Fusion

When retrovirus-producing and -susceptible cells are mixed, viral Env proteins on the cells can effectively interact with infection receptors on the neighboring susceptible cells via direct cell-to-cell contact. The interactions can have both positive and negative effects on the retrovirus replication. First, they can lead to cell-to-cell infection that allows very rapid and synchronized replication of virus compared to the cell-free infection [25, 26]. This can be advantageous for the virus replication in the presence of antiviral agents [27]. Second, the interactions can induce a negative effect, that is, the rapid apoptotic cell death, via syncytium formation [28–30]. This can be disadvantageous for the virus in that the sustained production of progeny virions becomes impossible. If the apoptotic cell death proceeded more efficiently than the virus replication, it eventually would result in poor progeny virus production. Therefore, it is conceivable that the retroviruses have some mechanisms to attenuate fusion capability of the envelope TM proteins in virus-producing cells and to primarily activate it in retroviral particles upon virion budding. Consistently, such mechanisms have been suggested for the Env TM proteins of MLV and HIV.

In the case of MLV Env protein, C-terminal 16-amino acid peptide of the TM subunit called R peptide is further cleaved by the retroviral protease after the budding [31, 32]. The R peptide-containing Env protein is expressed in the virus-producing cells. The R peptide-truncated MLV Env protein can induce syncytia in susceptible cells, but the R peptide-containing Env protein cannot, indicating that the R peptide negatively regulates the syncytium formation of virus-producing cells [33, 34]. Viral particles carrying the R peptide-containing Env protein have much lower infectivity

Table 1: Inhibitors used in studies of retroviral entry pathway.

Inhibitors	Target
Ammonium chloride	Acidification of intracellular vesicles
Bafilomycin A-l	Acidification of intracellular vesicles
Concanamycin A	Acidification of intracellular vesicles
Dynasore	Dynamin-dependent endocytosis
Chlorpromazine	Clathrin-dependent endocytosis
CA-074Me	Cathepsin B protease
Dynamin DN mutant[1]	Dynamin-dependent endocytosis
Caveolin DN mutant	Caveolin-dependent endocytosis
Clathrin DN mutant	Clathrin-dependent endocytosis
Eps 15 DN mutant	Endocytosis

[1] DN: dominant negative.

than those with the R peptide-cleaved Env, showing that the R peptide cleavage during virion maturation is required for the infectivity [35–37]. It has been reported that the R peptide controls the three-dimensional structure of the SU protein [38] and a disulfide bond between the SU and TM proteins [39], suggesting that the R peptide of TM subunit regulates the receptor-mediated SU conformational changes through the S–S bond between the SU and TM. It has been recently shown that the R peptide-cleaved TM forms separated Env legs, but the R peptide ties the TM legs together [40].

Although the C-terminal domain of the HIV TM protein is not cleaved, it is suggested that interaction between the HIV TM C-terminal region and Gag precursor protein suppresses the membrane fusion activity in virus-producing cells [41]. Processing of the HIV Gag precursor after budding abrogates the suppression of membrane fusion, and the mature virions gain sufficient fusion activity for the entry. The functions of C-terminal tails of retroviral Env proteins to inhibit membrane fusion are conserved among many retroviruses [42–45], though the mechanisms are different. The C-terminal domains of retroviral Env glycoproteins function to maintain the production of progeny virions by suppressing syncytium formation-directed apoptosis of virus-producing cells.

5. PH-Dependent Retrovirus Infection

Ammonium chloride, a weak base, neutralizes acid conditions in intracellular vesicles (Table 1). Concanamycin A and bafilomycin A-1 are specific inhibitors of the ATP-dependent proton pump/vacuolar ATPase (V-ATPase) that serves to acidify endocytic vesicles [46, 47]. To analyze the pH dependence of retrovirus entry, these compounds are frequently used. Additionally these inhibitors may affect trafficking of the intracellular vesicles, because siRNA-mediated knockdowns of subunits of V-ATPase complex affect trafficking of intracellular vesicles [48]. Previously it had been reported that ammonium chloride inhibits ecotropic MLV infection but does not amphotropic and xenotropic MLV infections, showing that ecotropic MLV infection occurs through acidic vesicles, but amphotropic and xenotropic MLV infections do not [49, 50] (Table 2). The more specific inhibitors of endosome acidification (concanamycin A and bafilomycin A-1) suppress all of ecotropic, amphotropic, polytropic, and xenotropic MLV infections [51, 52]. At present, it is generally accepted that ecotropic MLV infection requires acidification, because all the studies consistently reported the suppression of ecotropic virus replication with the inhibitors of endosome acidification. In contrast, it has been shown that xenotropic MLV infections are not suppressed by bafilomycin A-1 [53] (Table 2). Due to the controversial results, the entry pathway of xenotropic MLV is not clear yet. Because different cell lines were used in those reports, the low pH requirement of the xenotropic MLV infection may be dependent on the used cell lines (see below).

In case of avian leukosis virus (ALV) infection, there are also several controversial reports. The earlier reports show that ammonium chloride and bafilomycin do not affect ALV infection, suggesting that ALV infection does not require the acidification [54, 55]. In contrast, it has been recently reported that lowering the pH results in quick and extensive cell-cell fusion by ALV [56] and that the acidification inhibitors suppress ALV infection [57, 58]. It is now thought that receptor binding of ALV induces the Env protein to convert to its prehairpin intermediate at neutral pH [59, 60], and then endosome acidification triggers the formation of the final fusion-active form of the Env protein [61–63]. It has been proposed that the discrepancy came from unusual stability of the Env prehairpin intermediate, consequent ability of fusion to proceed upon washout of the acidification inhibitors after several hours, and the relatively high pH requirement for the outer leaflet mixing [64]. Finally, it is considered that ALV entry requires endosome acidification.

The acidification inhibitors suppress infections by mouse mammary tumor virus (MMTV) [65], foamy virus [66], equine infectious anemia virus (EIAV) [67, 68], Jaagsiekte sheep retrovirus (JSRV) [69], and enzootic nasal tumor virus [70]. These results suggest that infections by many animal retroviruses are low pH dependent.

6. Internalization Pathways

The requirement of low pH for the retrovirus infections reveals that retrovirus particles are internalized into acidic intracellular compartments during virus replication. There are several different pathways for the internalization of molecules; (i) phagocytosis, (ii) macropinocytosis, (iii) clathrin- and dynamin-dependent endocytosis, (iv) caveolin- and dynamin-dependent endocytosis, (v) lipid raft- and dynamin-dependent endocytosis, (vi) clathrin-, caveolin-, and dynamin-independent endocytosis that requires lipid raft, and (vii) dynamin-, clathrin-, caveolin-, and lipid raft-independent endocytosis [48, 71]. Here we will briefly summarize the accepted mechanisms and roles of internalization, relevant to the present review [48, 72, 73].

6.1. Phagocytosis. Specialized cells such as macrophages, neutrophils, and monocytes clear debris and pathogens

Table 2: Differential dependence of HIV and MLV infections on endosome acidification.

Viruses	Dependence of acidification	Cell lines	Reference
Ecotropic MLV	Independent	Rat XC	[49, 52]
	Dependent	Mouse NIH3T3, human TE671	[49, 51, 52]
Amphotropic MLV	Independent	Mouse NIH3T3, rat XC	[49, 52]
	Dependent	Mouse NIH3T3, human TE671	[51, 52]
Polytropic MLV	Independent	Rat XC	[52]
	Dependent	Mouse NIH3T3, human RE671, rat XC	[52]
Xenotropic MLV	Independent	Human HT1080, HTX, porcine, rat XC	[49, 50, 52, 53]
	Dependent	Mouse NIH3T3, human RE671	[52]
CD4-dependent HIV	Independent	Human CEM, HeLa, C8166, VB	[49, 89–93]
	Independent	Human 293T, HeLa, TE671	[21]
CD4-independent HIV	Dependent	Human 293T, HeLa, TE671	[21]

by phagocytosis. Signaling cascades induce the actin rearrangement and form membrane extensions that cover the target particles and engulf it. Phagosomes become acidic by fusion with lysosomes (pH 5.0-6.0). Debris internalized by phagocytosis is degraded in the acidic phagosomes (phagolysosomes).

6.2. Macropinocytosis. Stimulation by certain growth factors or other signals causes membrane protrusions that fuse with the plasma membrane to form large intracellular vesicles known as macropinosomes that encapsulate large volumes of the extracellular fluid. Macropinosomes can either fuse with lysosomes (pH 5.0-6.0) or recycle back to the cell surface. There is no consensus as to the final fate of macropinosomes. Trafficking of macropinosomes seems to depend on cell type and mode of macropinocytosis induction.

6.3. Clathrin-Mediated Endocytosis. After ligands bind to their receptors, the receptor proteins are internalized into intracellular vesicles called endosomes. The endosome formation requires dynamin GTPase, and the endosomes are coated by clathrin proteins. Many receptors are segregated from their ligands in early endosomes due to weakly acidic condition (pH 6.0). Early endosomes become more acidic by V-ATPase-mediated acidification (late endosomes/lysosomes) (pH 5.0-6.0), and separated ligands are degraded by endosome proteases. Certain receptors are transferred from early endosomes to recycling endosomes (pH 6.4) and are reused on the plasma membrane. Some proteins are also recycled from late endosomes/lysosomes through the trans-Golgi network. Lysosomes often form multivescular bodies.

6.4. Caveolin-Mediated Endocytosis. Glycosylphosphatidylinositol (GPI)-anchored proteins, simian virus 40 (SV40), and cholera toxin trigger the formation of caveolae coated by caveolin proteins. These ligands are internalized into intracellular vesicles (pH 7.0) dependently on dynamin GTPase. The vesicles can be sorted to endosomes and become acidic.

6.5. Clathrin- and Caveolin-Independent Endocytosis. Cholera toxin and SV40 can also be internalized via raft microdomains into GPI-anchored protein-enriched endosomes. Mechanisms regulating this internalization pathway are unclear as of yet.

7. Internalization of Retroviral Particles into Intracellular Vesicles

A dominant negative mutant of caveolin [74], siRNA-mediated knockdown of dynamin, and a dynamin inhibitor (dynasore) (Table 1) [52] suppress the amphotropic MLV infection, suggesting that amphotropic MLV particles are internalized by the dynamin- and caveolin-dependent endocytosis for productive infection (the fourth pathway). Ecotropic MLV particles are internalized into intracellular vesicles, but the vesicles are not colocalized with clathrin [75]. Furthermore, the dynamin-dominant negative mutant does not inhibit ecotropic MLV infection in human HeLa cells expressing the ecotropic MLV receptor, suggesting that ecotropic MLV particles are internalized by clathrin- and dynamin-independent endocytosis [75]. In contrast, another report indicates that siRNA-mediated knockdown of dynamin and dynasore suppresses ecotropic MLV infection in mouse NIH3T3, rat XC, and human TE671 cells expressing the ecotropic receptor [52] (Table 3). As mentioned above, the internalization pathway of ecotropic MLV might be dependent on the cell lines used. ALV [76] and EIAV [77] infections occur through clathrin-dependent endocytosis. JSRV infection required dynamin-dependent endocytosis [69]. Taken together, these reports strongly support a notion that infections by many animal retroviruses occur through endosomes and require endosome acidification.

All of intracellular vesicles do not necessarily become acidic. For example, macropinosomes can be recycled to plasma membrane before their acidification, and recycling endosomes are formed from early endosomes and are transferred to plasma membrane [48]. Because many retroviral infections require endosome acidification, if viral particles are internalized into recycling endosomes, infectivity would decrease. To prevent this, the interaction between retrovirus

Table 3: Differential internalization pathways of HIV and MLV infections.

Viruses	Internalization pathway	Cell lines	Reference
Ecotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
	Dynamin-, clathrin independent	Human HeLa	[75]
Amphotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
	Caveolin dependent	Mouse NIH3T3	[74]
Polytropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
Xenotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
CD4-dependent HIV	Dynamin dependent	Human HeLa	[95]
	Clathrin dependent	Human primary T lymphocyte	[95–97]
	Dynamin-, Eps15 dependent	Human HeLa	[98]
	Dynamin-, Eps15 independent	Human 293T, HeLa, TE671	[21]
CD4-independent HIV	Dynamin-, Eps15 dependent	Human 293T, HeLa, TE671	[21]

Env proteins and the infection receptors is speculated to induce a signal to trigger the acidification of virion-containing intracellular vesicles.

8. Cleavage of Retroviral Env Proteins by Cathepsins

Many retrovirus infections require endosome acidification. Influenza virus infection also requires endosome acidification, and treatment of influenza virus particles with low pH buffer activates its membrane fusion, indicating that low pH treatment directly induces conformational change of the influenza virus hemagglutinin to the fusion-active form. In contrast, low pH treatment of MLV particles does not activate the membrane fusion. Why does ecotropic MLV entry require endosome acidification?

There is another mystery of the endosome-mediated infection. Proteins internalized into acidic late endosomes/lysosomes are generally degraded by endosome proteases including cathepsins. The acidification inhibitors suppress the degradation in late endosomes/lysosomes [47]. If the retroviral particles are degraded in late endosomes/lysosomes, the acidification inhibitors would enhance retrovirus infection. However, the acidification inhibitors rather suppress the infection [52]. Therefore, it is suggested that the retroviral particles incorporated into late endosomes/lysosomes are not degraded. Why are the retroviral particles not degraded in acidic late endosomes/lysosomes?

The finding that endosomal cathepsin proteases are necessary for the ecotropic MLV infection [78, 79] like Ebola virus infection [80] has provided a clue to understanding the questions. Because cathepsin proteases are activated by acidification, the ecotropic MLV entry into host cytoplasm requires cathepsin activation by acidification. The weakly acidic condition (pH 6) in early endosomes cannot activate cathepsin proteinases [81], suggesting that ecotropic MLV infection occurs via late endosomes/lysosomes. The acidification inhibitors suppress MLV infections by attenuating cathepsin protease activation. The evidence that the acidification inhibitors do not suppress the ecotropic MLV infection in active cathepsin-containing medium further

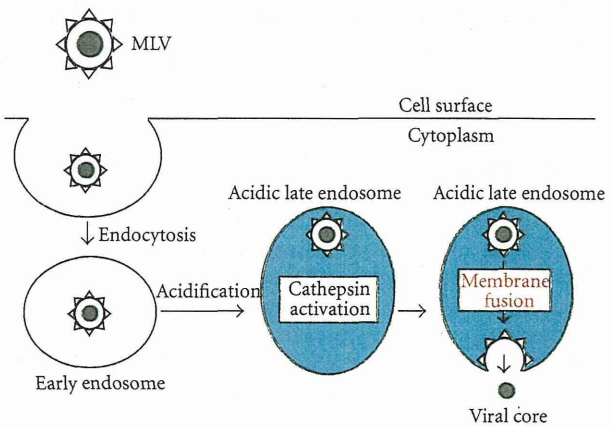

Figure 2: Entry pathway of ecotropic MLV in almost all susceptible cells. Blue area indicates acidic condition.

supports this conclusion [52]. Our current model for entry of ecotropic MLV is that cathepsin proteases digest MLV Env glycoproteins to generate fusion-active forms rather than to break them up completely, because treatment of ecotropic and amphotropic MLV particles with cathepsin B protease results in a few digested products of the Env proteins but not their disappearance [52, 79]. It is still unclear how the MLVs are not degraded in the late endosomes/lysosomes by other proteases.

In summary, the entry pathway of ecotropic MLV occurs as follows (Figure 2). Ecotropic MLV particles are internalized into endosomes, following the interaction of Env protein with the infection receptor. The viral particle-containing endosomes become acidic by V-ATPase. Cathepsin proteases are activated in the acidic late endosomes. The activated cathepsins cleave the ecotropic Env proteins to confer them fusion active. The cleaved Env proteins induce fusion between the viral envelope and host cell endosome membranes. Finally, the ecotropic MLV cores enter into host cytoplasm.

Although it is widely accepted that the ecotropic MLV infection requires endosome acidification and cathepsin proteases, the entry pathway of xenotropic MLV is not clear,

because of the contradictory reports [52, 53]. We have shown that xenotropic MLV infection requires endosome acidification and cathepsin proteases like the ecotropic MLV infection [52]. In sharp contrast, the Liu research group has reported that inhibitors of endosome acidification and cathepsin proteases do not inhibit the xenotropic MLV infection [53]. Different cell lines used in these studies may induce different entry pathways of the xenotropic MLV.

Unlike the ecotropic MLV entry, it has been reported that a low-pH pulse of JSRV particles overcomes the bafilomycin-mediated infection inhibition [69], EIAV infectivity is enhanced by low-pH treatment [67], and cell-cell fusion induced by the ALV Env protein is enhanced at low pH [55]. Additionally, analysis of the pH dependence of the foamy virus Env-mediated fusion in a cell-cell fusion assay revealed an induction of syncytium formation by a short exposure to acidic pH [66]. The low-pH treatment of these retroviruses may directly induce the conformational changes of their Env glycoproteins to fusion active forms without the proteolytic cleavage, like influenza virus.

9. PH-Independent MLV Infection in XC Cells

Although the acidification inhibitors attenuate the ecotropic MLV infection in almost all susceptible cells [49, 52], the inhibitors have no effect on the ecotropic MLV infection specifically in rat XC cells, suggesting that the ecotropic MLV infection in XC cells is independent of low pH [49] (Table 2). Furthermore, the R peptide-containing ecotropic Env protein can induce pH-independent syncytium formation in XC cells, but cannot in other susceptible cells [82, 83]. By these results, it had been widely thought that ecotropic MLV entry into XC cells occurs at cell surface membranes and does not require the internalization of virions into intracellular vesicles and acidification. This XC cell-specific pH-independent ecotropic MLV infection was one of the well-known mysteries in the MLV field [84, 85]. We found that a cathepsin inhibitor, CA-074Me, efficiently suppresses the ecotropic MLV infection in XC cells, like in other susceptible cells, suggesting that the ecotropic MLV infection in XC cells requires endosomal cathepsin proteases [52]. This result is inconsistent with the previous theory that the ecotropic MLV infection in XC cells does not occur through endosomes. Because the ecotropic MLV infection requires cathepsin proteases activated by endosome acidification, the acidification inhibitors would be proposed to suppress the MLV infection by attenuating cathepsin activation. However, the acidification inhibitors do not reduce cathepsin activity in XC cells, but do so in other cell lines, suggesting that cathepsin proteases are activated without endosome acidification in XC cells [52]. XC cells do not express so much cathepsin that activation is sufficient at suboptimal pH, because cathepsin activity of XC cells is comparable to that of NIH3T3 cells. These results prompted us to speculate that the ecotropic MLV infection in XC cells occurs through endosomes. The result that dynasore and siRNA-mediated knockdown of dynamin expression suppress the ecotropic MLV infection in XC cells strongly supports this hypothesis.

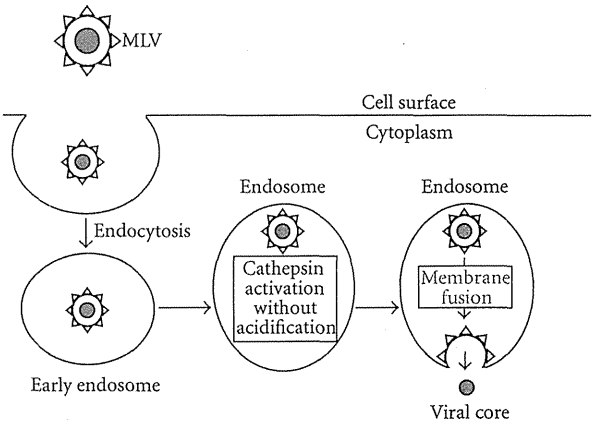

FIGURE 3: Entry pathway of ecotropic MLV in XC cells. Ecotropic MLV entry in XC cells may occur in acidic late endosomes, but endosome acidification is not required for the entry.

Taken together, the entry pathway of ecotropic MLV in XC cells is considered as follows (Figure 3). The ecotropic MLV particles are internalized into endosomes in XC cells, like in other susceptible cells. Cathepsin proteases are activated without endosome acidification. The activated cathepsins cleave the MLV Env protein, and the fusion between the viral envelope and host cell endosome membrane takes place for entry of the viral core into host cytoplasm. Because of the endosome acidification-independent activation of cathepsin proteases [52], the acidification inhibitors do not suppress the cathepsin protease activity and ecotropic MLV infection in XC cells. Additionally, this finding supports the above-mentioned hypothesis that the acidification inhibitors differentially affect retrovirus infections in different cell lines. The mechanism of acidification-independent cathepsin activation in XC cells is waiting to be resolved.

10. PH-Dependent Entry and PH-Independent Syncytium Formation by Retroviral Env Proteins

The R peptide-cleaved MLV Env protein induces the fusion between the viral envelope and host cell membranes for viral entry and syncytium formation in susceptible cells [33, 34]. Cells expressing the R peptide-truncated Env protein behave as large MLV particles and fuse with neighboring susceptible cells. Therefore, the syncytium formation by the retroviral Env proteins is thought to represent the membrane fusion in retroviral entry. Because the syncytium formation by the retroviral Env protein may contribute to the development of degenerative disorders like AIDS [28, 29], and because an endogenous retroviral Env protein (syncytin) induces syncytiotrophoblast formation [86], the elucidation of mechanism of retroviral Env-induced syncytium formation is essential to understand retroviral pathogenesis and placenta development. The MLV entry into host cells is dependent on low pH, but the syncytium formation by the R peptide-truncated Env protein is independent [33].

Furthermore, the viral envelopes fuse with host cell membrane in endosomes [52, 75], but the syncytium formation appears to result from the fusion of cell surface membranes of the Env-expressing and host cells. In addition, the Env glycoprotein of a CD4-independent HIV efficiently induces pH-independent syncytium formation [87], but infection by CD4-independent HIV occurs through acidic endosomes [21] (see below). Multiple interactions between the viral Env and infection receptor proteins in much larger areas of cell-cell contact than virus-cell contact may abrogate the requirement of endocytosis for the membrane fusion. The finding that a cell adhesion molecule, LFA-1, facilitates HIV-mediated syncytium formation but not HIV infection supports this idea [88]. If the syncytium formation by the Env protein is independent of endocytosis, cathepsin proteases would be unnecessary for the syncytium formation. However, cathepsin inhibitors suppress syncytium formation by the ecotropic MLV Env protein [79]. Secreted cathepsin proteases may be involved in the pH-independent syncytium formation by the Env protein. Further study is needed to understand the mechanism of pH-independent syncytium formation by the retroviral Env proteins.

11. Endocytic Pathway of CD4-Dependent and -Independent HIV Entry

There are many controversial reports of the role of endocytosis in CD4-dependent HIV infection [94] (Tables 2 and 3). Early reports indicate that the acidification inhibitors enhance [89–91] or do not affect CD4-dependent HIV infection [92, 93], suggesting that the HIV does not enter into host cells via acidic vesicles. However, recent reports show that dynasore and chlorpromazine attenuate CD4-dependent HIV infection [95–97]. In addition, dominant negative mutants of dynamin and Eps15 inhibit CD4-dependent HIV infection [98]. Furthermore, analysis of localization of labeled HIV particles revealed that the HIV particles are internalized into intracellular vesicles [95, 99–102]. It has been reported that envelopes of HIV particles fuse with host cell membranes in intracellular vesicles by the following observation [95]. Envelopes of HIV particles were labeled with a hydrophobic fluorescent compound. When fusion of the labeled HIV envelope with host cell membrane occurs, the fluorescent compound is diluted and the fluorescent signals disappear. The vanishing of the fluorescent signals was observed in the intracellular vesicles but not at cell surfaces. These results suggest that HIV entry into the host cell cytoplasm may occur via endosomes.

Interestingly, endosome acidification inhibitors attenuate infections by CD4-independent HIVs, which are thought to be prototypes of CD4-dependent viruses, suggesting that CD4-independent HIV entry may occur through acidic late endosomes, like many animal retroviruses [21]. The CD4-dependent HIVs can infect CD4-negative trophoblastic cells though the infection is 100 times less efficient than CD4-dependent Env-mediated infection [103]. HIV infection of trophoblasts forming the placental barrier may cause the mother-to-child transmission of HIV [104]. This infection occurs through an unusual entry pathway that is clathrin-, caveolin-, and dynamin-independent endocytosis requiring free cholesterol [71].

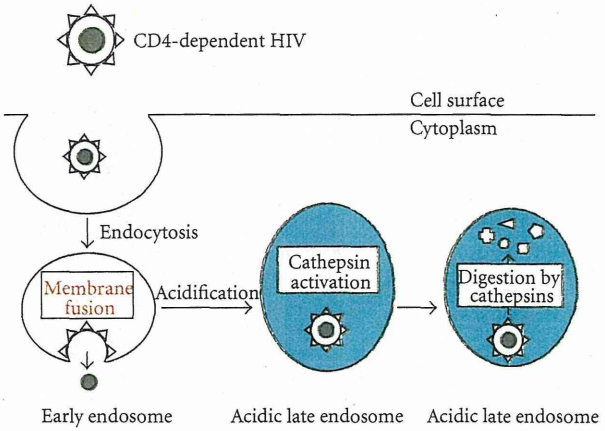

FIGURE 4: Entry pathway of CD4-dependent HIV. Blue area indicates acidic condition.

12. Degradation of HIV Particles by Endosome Proteases

Because acidification inhibitors enhance CD4-dependent HIV infection [89–91], HIV entry is independent of low pH, and the viral particles internalized into acidic late endosomes are degraded [105]. In other words, a proportion of HIV particles are internalized into acidic late endosomes although the internalization into late endosomes is not associated with the HIV productive infection. Consistently, the HIV particles appear to be internalized into acidic compartments shortly after inoculation into host cells [100].

In summary, entry pathway of CD4-dependent HIV is considered as follows (Figure 4). The HIV particles are internalized into host cells by endocytosis, and the entry is independent of endosome acidification. HIV entry mainly occurs at early endosomes, and the HIV particles internalized into acidic late endosomes are degraded by endosome proteases.

It has been reported that a cathepsin inhibitor CA-074Me more significantly enhances CD4-independent HIV infection than CD4-dependent infection, and cathepsin protease activity in host cells is reverse-correlated with cellular susceptibility to the CD4-independent HIV infection [21]. These results suggest that CD4-independent HIV entry may occur at acidic late endosomes, and that viral entry competes with virion degradation by cathepsin proteases (Figure 5).

Degradation by endosomal proteases in acidic vesicles following phagocytosis/macropinocytosis/endocytosis functions as an innate immune reaction against microbes to digest them and generate antigen peptides presented to helper T cells on MHC class II [106]. In fact, the activation of toll-like receptor signaling by LPS enhances cathepsin expression [21]. The CD4-dependent HIVs might evolve

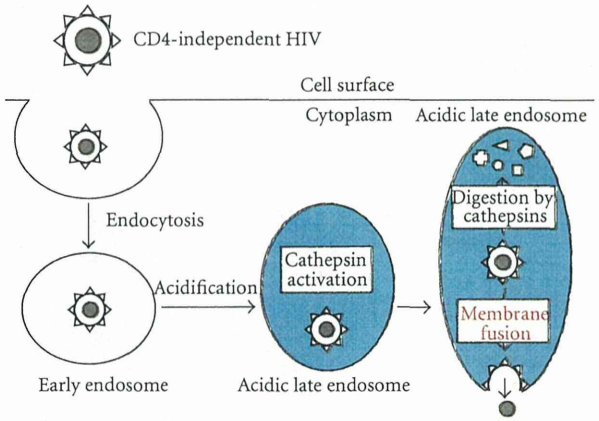

FIGURE 5: Entry pathway of CD4-independent HIV. Blue area indicates acidic condition.

from CD4-independent viruses to overcome the endosome protease-mediated immunity. Some microbes express cystatin-like cathepsin inhibitors to protect themselves from the cathepsin-mediated immunity [107, 108]. Instead of having a cathepsin inhibitor, the CD4-dependent HIVs might gain the acidification-independent entry mechanism to protect from the endosome protease-mediated immunity.

In contrast to the CD4-dependent HIV entry pathway, ecotropic MLVs utilize these cellular innate immune reactions of endocytosis, acidification, and digestion by endosome proteases to enter into the host cell cytoplasm. By the ecotropic virus entry mechanism, the viruses can escape from these host immune reactions. It is suggested that the CD4-dependent HIV entry utilizes endocytosis, but not acidification and proteolysis by endosome proteases. The CD4-dependent HIV particles may be degraded by endosome proteases in acidic endosomes, and the infection titer is reduced [89, 91]. The CD4-dependent HIV Env proteins indeed contain several amino acid motifs that are digested by cathepsins [109, 110]. The ecotropic MLVs also have cathepsin-recognized amino acid motifs, but the digestion may activate the membrane fusion capability of the Env protein.

As mentioned above, the cathepsin inhibitor enhances CD4-independent HIV infection in cells with relatively higher level of cathepsin protease activity [21]. While, treatment of such cells with CA-074Me at higher concentration attenuates the CD4-independent infection. In addition, CA-074Me suppresses the CD4-independent HIV infection in cells with lower cathepsin activity (unpublished data). These results suggest that cathepsin proteases are required for the CD4-independent infection. Therefore, Env glycoproteins of the CD4-independent HIVs may be digested by cathepsin proteases to a fusion-active form, like the ecotropic MLV Env protein. Consistently, cathepsin proteases enhance CD4-dependent HIV infection and confer CD4-negative cells susceptible to CD4-dependent HIV infection [111–113]. Cathepsin-mediated digestion of CD4-dependent HIV Env protein may induce membrane fusion without CD4 binding. HIV particles in acidic endosomes are degraded by many endosome proteases including cathepsins. However, when the HIV Env proteins are digested only by a cathepsin, the infectivity may be enhanced.

13. Entry of Targeted Retroviral Vector

Retroviral vectors are valuable tools in molecular biology research and human gene therapy. Several fundamental properties of retroviral vectors remain to be improved for effective gene transfer to specific target cells [114]. The effectiveness will be greatly enhanced, if their infection tropism is artificially modified to target specific cells [115]. There have been various attempts to establish redirecting infection tropism by genetically incorporating heterogenous ligands into the retroviral Env proteins [116–121]. However, retroviral vectors containing such modified Env proteins suffer from very low transduction efficiency or are not infectious. The redirected transductions of retroviral vectors with chimeric Env proteins are enhanced by the endosome acidification inhibitors, suggesting that the targeted vector particles internalized into acidic endosomes are degraded by endosome proteases [120, 122].

Retroviral vectors carrying the ecotropic Env proteins chimeric with SDF-1α [123] and somatostatin [124] can transduce cells expressing CXCR4 and somatostatin receptor, respectively, as efficiently as retroviral vectors with the wild-type Env protein. It has not been examined whether efficient infections by the redirected retrovirus vectors occur through endosomes. Because the SDF-1α-chimeric Env protein appears to induce infection by the same mechanism as the wild-type Env protein [125], the redirected infection may occur through endosomes and require endosome acidification, like the wild type MLV Env protein. Elucidation of the entry pathways of these targeted retroviruses will likely contribute to the development of efficient cell lineage-specific retrovirus vectors.

14. Endocytic Entry of Ebola Virus-Pseudotyped Retrovirus Vector

Retrovirus vectors can be pseudotyped with glycoproteins of various enveloped viruses. The pseudotyped retrovirus vectors enter into host cells by the entry mechanisms of the heterologous viral glycoproteins. Because the retrovirus vectors do not produce replication-competent viruses and the protocol is relatively simple, pseudotyped retrovirus vectors are widely used to identify entry pathways of various enveloped viruses [126–128].

A dominant negative mutant of Eps15, siRNA-mediated knockdown of clathrin, and chlorpromazine suppress infection by an HIV vector pseudotyped with Ebola virus glycoprotein (GP), indicating that Ebola virus GP-mediated entry occurs through clathrin-dependent endocytosis [129]. Virion morphologies of the pseudotyped HIV vector and Ebola virus are much different. The pseudotyped HIV vector particles are round and the diameter is around 100 nm regardless of viral envelope glycoproteins. Whereas Ebola

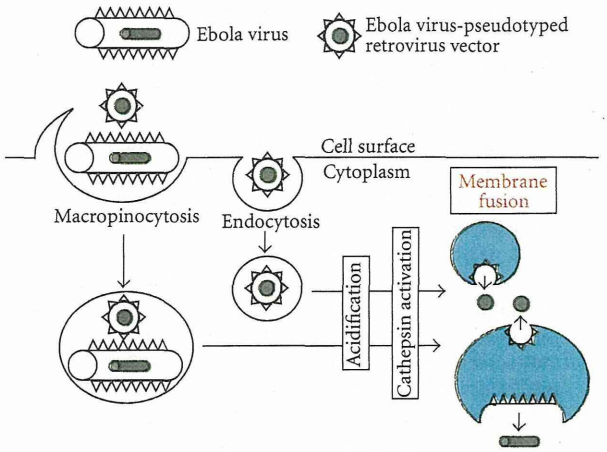

FIGURE 6: Entry pathways of Ebola virus and Ebola virus-pseudotyped retrovirus vector. Blue area indicates acidic condition.

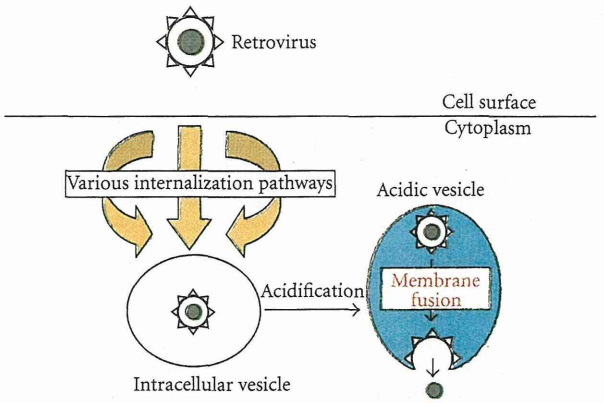

FIGURE 7: Retrovirus particles are internalized into intracellular vesicles by various pathways, and vesicle acidification is necessary for the infections.

virus virions are long and filamentous as the name of filovirus should show. Typical clathrin-coated vesicles are large enough to incorporate the HIV vector particles, but not Ebola virus particles. Therefore, Ebola virus particles cannot be internalized into the endosomes. Does Ebola virus enter into host cells through endosomes? The finding that Ebola virus entry occurs via macropinosomes resolved this problem [130–133] (Figure 6). Macropinosomes have enough size to incorporate Ebola virus particles. However, entry of intact Ebola virus is still dependent on dynamin, which is not involved in classical macropinocytosis [133], and is partially inhibited by inhibitors of clathrin-dependent endocytosis [132]. In addition, it has been reported that the Ebola virus entry through macropinocytosis or endocytosis is dependent on the cell lines used [134]. Therefore, the entry route of Ebola virus is not clear yet. The Ebola virus infections via endocytosis and macropinocytosis both require acidification and cathepsin proteases [80, 135]. Although the pseudotyped retrovirus vector is useful to study the entry mechanism of viral envelope proteins, we should notice the possibility that entry pathway of the pseudotyped retrovirus vector is different from that of the original virus.

Size of macropinosomes is enough to incorporate not only Ebola virus particles but also pseudotyped HIV vector particles. Therefore, Ebola virus-pseudotyped HIV vector entry can occur through macropinocytosis (Figure 6). There is a report showing that HIV infection occurs through macropinosomes [102]. If host cells have both dynamin-independent macropinocytosis and -dependent endocytosis, the inhibition of dynamin function does not significantly affect the pseudotyped HIV vector infection. If host cells have endocytosis but not macropinocytosis, the inhibition of dynamin function severely suppresses the pseudotyped HIV vector infection. Retrovirus entry may be able to occur through several distinct internalization pathways for productive infection (Figure 7). This may be the reason why the inhibitors differentially affect retrovirus infections in different cells. Pathways of retrovirus internalization into intracellular vesicles may be unimportant for the productive infection. The GP of Ebola virus that enters host cells via macropinosomes can use endocytosis for the productive entry, when the retrovirus vector is pseudotyped with the Ebola virus GP. This result strongly supports the idea.

15. Conclusion

Infections by many animal retroviruses occur through endosomes and require endosome acidification. The activation of cathepsin proteases by endosome acidification is required for ecotropic MLV infection. Whereas acidification directly induces conformational changes of several retroviral Env proteins to the fusion active forms. There are several internalization pathways of retrovirus particles, and the viral internalization pathways appear to be different in different cell lines. CD4-independent HIV infection may occur through endosomes and require endosome acidification, like other animal retroviruses. CD4-dependent HIV infection is thought to occur through endosomes but does not require endosome acidification. The CD4-dependent and -independent HIV particles are both degraded by endosome proteases, when the viral particles are internalized into acidic late endosomes. Retrovirus vectors pseudotyped with other viral envelope proteins are widely used to understand the entry mechanisms of the envelope proteins. However, entry pathway(s) of the pseudotyped retroviral vector could be different from that of the original virus.

Retroviruses require cellular biological events of internalization, vesicle acidification, and cathepsin proteolysis for their entry into host cells. These biological events, especially in phagocytosis, function to protect host cells from microbe infection. Retroviruses utilize these immune reactions to enter into host cells. This entry mechanism of retroviruses is the best strategy to overcome the host immune attack, and many viruses other than retroviruses also enter into host cells by similar mechanisms [72, 136].

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