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Negative regulation of the anti-human immunodeficiency virus and chemotactic activity of human stromal cell-derived factor 1α by CD26/dipeptidyl peptidase IV

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Abstract Stromal cell-derived factor 1α (SDF-1α) is a chemokine that has been shown to prevent infection of T-tropic HIV strains and is a possible substrate of CD26/dipeptidyl peptidase IV (DPPIV). In this study, we show that SDF-1α was cleaved at the N-terminal region by CD26/DPPIV and as a result the inhibitory activity of SDF-1α against HIV infection disappeared. Moreover, the chemotactic activity of SDF-1α also disappeared specifically by DPPIV activity of recombinant soluble CD26. These results suggested that dissemination of T-tropic HIV strains in vivo may be facilitated by CD26/DPPIV via inactivation of functional SDF-1α.

Key words: Anti-human immunodeficiency virus and chemotactic activity; CD26/dipeptidyl peptidase IV; Human immunodeficiency virus infection; N-terminal cleavage; Stromal cell-derived factor 1α

1. Introduction

CD26 is a widely distributed 110-kDa cell surface glycoprotein with known dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) activity in its extracellular domain. This ectoenzyme is capable of cleaving N-terminal dipeptides from polypeptides with either Pro or Ala residue in the penultimate position [1–4]. On human T cells CD26 expression appears late in thymic differentiation and is preferentially restricted to the $CD4^+$ helper memory cell population [2]. Previously we have shown that CD26 can deliver a potent co-stimulatory T cell activation signal and DPPIV activity is essential for CD26-mediated T cell co-stimulation [5,6]. From its cDNA sequence, CD26 is predicted to be a type II integral membrane protein with a large extracellular domain, transmembrane segment, and a cytoplasmic tail of six amino acids (aa), suggesting that in addition to DPPIV activity, other signal-inducing molecules may be associated with CD26 [7]. Previously, we have demonstrated that CD26 is physically associated, presumably in its extracellular domain, with CD45, a membrane-linked protein-tyrosine phosphatase [8]. More recent studies from our laboratory have shown that CD26 is identical to the adenosine deaminase (ADA)-binding protein [9–11]. CD45 and ADA are both thought to be important in regulation of T cell activation and signal transduction.

Chemokines play an important role in inflammation by attracting leukocytes [12]. Many kinds of chemokines and their receptors have been identified in the past few years, and importantly, it has been demonstrated that two of those chemokine receptors, namely CXCR4 and CCR5, function as coreceptors for infection of T-tropic (infects T cell lines) and M-tropic (infects macrophage and activated T cells) strains of HIV, respectively [13–19]. Subsequently, chemokines SDF-1 (stromal cell derived factor-1), a CXCR4 ligand, and RANTES (regulated on activation, normal T cell expressed and secreted) and MIP (macrophage inflammatory proteins) 1α and 1β, CCR5 ligands, were reported to block the cell entry of T-tropic and M-tropic HIV, respectively [20–22]. It is remarkable that several chemokines, including SDF-1α, RANTES and MIP-1β, have the conserved aa sequence for the substrate of DPPIV at the N-terminus, and T cells capable of transendothelial migration are those which express the highest levels of CD26 [23]. These observations have led us to the hypothesis that behavior of the chemokine might be modified by CD26/DPPIV. In this regard, it was shown that the receptor specificity and function of RANTES is regulated by CD26/DPPIV-mediated cleavage [24]. Recently, we reported that SDF-1 secreted from cells expressing CD26 molecules lost anti-HIV and chemotactic activity mediated by SDF-1 [25]. Therefore, experiments that more directly study the interaction between SDF-1 and CD26/DPPIV and its functional consequences were required.

In this study we examined whether SDF-1 is a substrate for CD26/DPPIV and the interaction of SDF-1 with CD26/DPPIV affects anti-HIV and chemotactic activity. We developed CD26-transfectants from Jurkat T cell line that originally does not express CD26 and recombinant soluble CD26s ($DPPIV^+$ and $DPPIV^-$) [26,27]. Here we directly demonstrate that CD26/DPPIV cleaves SDF-1α as a substrate and the cleavage of SDF-1α by CD26/DPPIV results in a loss of inhibition of HIV cell entry with SDF-1α. Moreover, we show that CD26/DPPIV causes a loss of chemotactic activity of SDF-1α. These results indicate that sensitivity against HIV infection and migratory degree of T lymphocytes in inflam-

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Abbreviations: A_{595}, optical absorbance at 595 nm; aa, amino acid(s); ADA, adenosine deaminase; CHO, Chinese hamster ovary; DPPIV, dipeptidyl peptidase IV; HIV, human immunodeficiency virus; mCD26, recombinant CD26 which lacks the majority of DPPIV activity; PBS, phosphate-buffered saline; RANTES, regulated upon activation, normal T cell expressed and secreted; sCD26, recombinant soluble CD26 having DPPIV activity; SDF-1α, stromal cell-derived factor 1α

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mation would be modulated by CD26/DPPIV via SDF-1α in vivo.

2. Materials and methods

2.1. Cell cultures

Murine fibroblast L cells were grown in minimum essential medium (MEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% FCS (MEM/FCS). Jurkat T cell line was grown in RPMI 1640 (Gibco-BRL) supplemented with 10% FCS, and the CD26-transfectants (DPPIV$^+$ and DPPIV$^-$) [27] were maintained in RPMI 1640 containing 10% FCS and 200 μg/ml geneticin (Gibco-BRL). Expression of CD4 and CXCR4 molecules on $>95\%$ of Jurkat cells and the transfectants were confirmed by FACSCalibur (Becton Dickinson, San Jose, CA, USA) analysis with monoclonal antibodies anti-CD4 (19thy) [27] and anti-CXCR4 (12G5; R&D Systems, Minneapolis, MN, USA), respectively. CD26 expression was also analyzed with monoclonal antibody 1F7 [2,27], and positive rates of parent Jurkat cells and its transfectants were 0.7% and 99%, respectively.

2.2. Generation of recombinant SDF-1 peptides

A *Not*I-fragment containing coding sequence of SDF-1α was subcloned into the *Not*I-site in pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) and resulted in pBS-SDF. To obtain recombinant plasmids for expression, five kinds of oligonucleotides were used for PCR amplification by Ex-Taq DNA polymerase (Takara, Japan): 5′-ATTGACTCTCTTCCGGGCGCTATCATG-3′ (named Ex-N), 5′-CTTGTCGTCGTCGTCGGTACCCAGA-3′ (Ex-EK), 5′-TGGCGAAAGGGGGATGTGCTGC-3′ (Ex-C2), 5′-TCTGGGTACCGACGACGACGACAAGAAACCAGTCAGCCTGAGCTACCGAT-3′ (Ex-S1), 5′-TCTGGGTACCGACGACGACGACAAGGTCAGCCTGAGCTACCGATGCCCCT-3′ (Ex-S2). A 0.6-kb DNA fragment amplified from pET-30a(+) (Novagen, Madison, WI, USA) using Ex-N and Ex-EK as primers, and a 0.5-kb DNA fragment amplified from pBS-SDF using Ex-S1 and Ex-C2 as primers, were purified from PCR products and mixed, and PCR was performed again using primers Ex-N and Ex-C2. The resulting 1.1-kb DNA fragment was digested with *Sph*I and *Xho*I, ligated with *Sph*I-*Xho*I digested pET-30a(+), yielding a SDF-1 encoding plasmid named pET-SDF1. Similarly, pET-SDF2 was constructed using Ex-S2 instead of Ex-S1 as a primer to produce the polypeptide in which 2 N-terminal amino acids (Lys-Pro) were deleted (2 aa-truncated SDF-1α). The nucleotide sequences of pET-SDF1 and pET-SDF2 were confirmed by analysis with ABI 373S sequencing system (Perkin Elmer, Japan).

Native and 2 aa-truncated SDF-1α were produced as S·tag fusion protein by pET expression system and purified by S·Tag fusion purification system (Novagen) according to the manufacturer's manual. Alternatively, *Escherichia coli* BL21 (DE3) harboring either pET-SDF or pET-SDF2 were solubilized with final concentration of 4 M urea and recombinant SDF-1α polypeptides were allowed to bind to S-protein agarose under 2 M urea condition. S·Tag fusion proteins bound to S-protein agarose were washed and treated with recombinant enterokinase (Novagen) at room temperature. After 16 h incubation enterokinase was removed by rEK capture agarose (Novagen) and recombinant SDF-1α peptides were eluted. Purified polypeptides were dialyzed with PBS and concentrated by Centricon-3 (Amicon, Beverly, MA, USA).

2.3. Cleavage of SDF-1α by sCD26

Recombinant soluble CD26 (sCD26) with DPPIV activity and mutant soluble CD26 (mCD26) which lacks DPPIV activity were prepared as described previously [26]. A specific DPPIV activitiy of sCD26 was 0.9 nmol *p*-nitroaniline released/min/μg whereas the activity of mCD26 was undetectable as determined by colorimetric assay using Gly-Pro-*p*-nitroaniline *p*-tosylate as a substrate. Five μg of native SDF-1α was mixed with 5 μg of sCD26 or mCD26 in 20 μl of PBS, and incubated at 37°C for 1 h. The reacted SDF-1α was separated with 20% SDS-polyacrylamide gel and electrotransferred to PVDF membrane for peptide sequencing. The N-terminal aa sequence was determined using HP G1005A protein sequencing system (Hewlett Packard, Palo Alto, CA, USA).

2.4. Cell-cell fusion assay

To clarify whether cell entry of HIV is achieved, we employed cell-cell fusion assay based on T7 RNA polymerase-dependent β-galactosidase expression [13]. As effector cells, mouse L cells were transfected with pET-30b(+) (Novagen) containing β-galactosidase gene using Lipofectamine (Gibco-BRL), followed by infection with recombinant vaccinia virus containing the envelope genes of HIV-1_{NL43} (Vac-Env_{NL43}). As responder cells, L cells were infected with recombinant vaccinia viruses containing human CD4 gene (Vac-CD4), bacteriophage T7 RNA polymerase gene (Vac-T7), and recombinant Sendai virus containing human CXCR4 gene (Sen-CXCR4) [28], while Jurkat T cells were infected with Vac-T7 only. Cells were infected at 10 multiplicity of infection for each recombinant virus. Cells were harvested after 24 h incubation at 37°C, washed with MEM/FCS twice, and resuspended with MEM/FCS. Responder cells were mixed with MEM/FCS containing recombinant SDF-1α and/or recombinant soluble CD26, and then effector cells were added. After 3 h incubation at 37°C, cells were lysed with 0.25% final concentration of Nonidet P-40. Fifty μl of cell lysate was added to 50 μl of substrate solution consisting of 120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 20 mM KCl, 2 mM $MgSO_4$, 10 mM 2-mercaptoethanol and 16 mM chlorophenol red-β-D-galactopyranoside, and the β-galactosidase activity was determined by change of A_{595}.

2.5. Migration assay

Isolation of human peripheral blood mononuclear cells and E rosette-positive (E$^+$) cells from healthy volunteers was described previously [2]. For further elimination of monocytes, E$^+$ cells were cultured in plastic dishes and non-adherent cells were used as a source of T cells throughout this experiment. Native or 2 aa-truncated SDF-1α with/without sCD26 or mCD26 were diluted in assay medium consisting of RPMI 1640 and 0.3% BSA, and incubated at 37°C for 1 h, followed by addition to culture plates in a final volume of 600 μl. Culture inserts (Transwell 3421, 5 μm pore size; Coster, Cambridge, MA, USA) were wetted with assay medium at 37°C for 1 h prior to use and transferred to culture plates, and 5×10^5 T cells resuspended in assay medium were added to each insert in a volume of 100 μl. Migration assay was performed by incubation at 37°C for 3.5 h. Cells migrated to the bottom chamber were harvested and counted using a Coulter EPICS XL flow cytometer for a set time period of 30 s [23].

3. Results and discussion

Although we showed indirectly that SDF-1 appears to be a substrate of CD26/DPPIV [25], there still is a possibility that other cellular components may be involved in the cleavage of SDF-1. Therefore, we first determined whether CD26/DPPIV cleaves SDF-1α without other factors in vitro. For this purpose, we have developed recombinant soluble CD26s (DPPIV$^+$ and DPPIV$^-$) secreted from transfected CHO cells [24]. As a result, N-terminal sequencing of SDF-1α after incubation with sCD26 revealed that SDF-1α was cleaved in front of the third Val residue (Val-Ser-Leu-Ser-Tyr was determined as the N-terminal aa sequence), whereas the untrun-

Table 1
Effect of sCD26 DPPIV activity on chemotaxis with SDF-1α

Assay medium containing:	None	sCD26	mCD26
None	827 ± 127	675 ± 36	668 ± 130
Native SDF-1α	1309 ± 251	706 ± 49	1605 ± 301

Values indicate means of transmigrated T cell count and standard errors obtained from triplicate experiments. Final concentration of 50 ng/ml for SDF-α and 660 ng/ml for sCD26 or mCD26 were added (1:1 of molar ratio).

cated N-terminal aa sequence (Lys-Pro-Val-Ser-Leu) of SDF-1α was detected when SDF-1α was incubated with mCD26, a DPPIV-defective form of sCD26 (data not shown). This result clearly indicated that SDF-1α is cleaved as a physiological substrate of CD26/DPPIV.

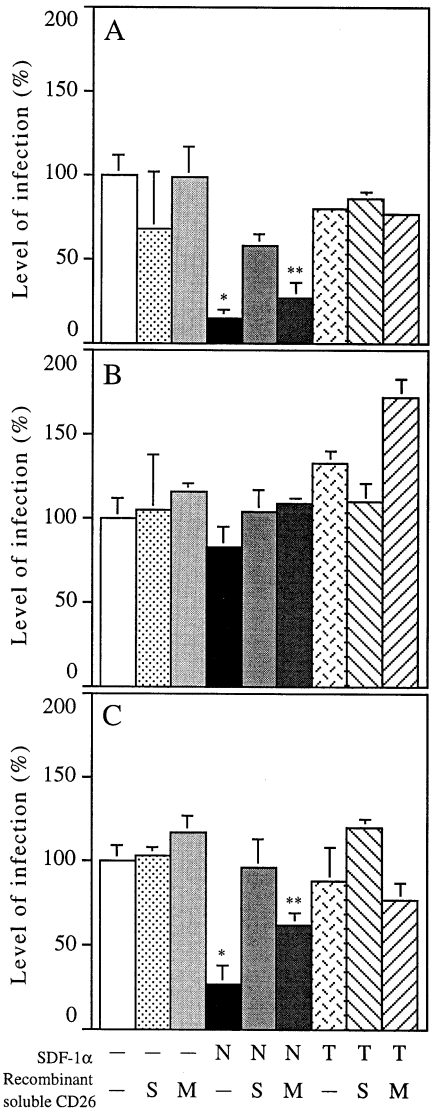

Fig. 1. Inhibition of CXCR4-mediated infection of HIV-1_{NL43} by native and 2 aa-truncated SDF-1α, monitored with β-galactosidase activity dependent on cell-cell fusion. 3×10^5 cells of parent Jurkat T cell line (A), wild-type CD26 (DPPIV$^+$) transfectant (B) and mutant CD26 (DPPIV$^-$) transfectant (C) were infected with Vac-T7, and then incubated with 1×10^5 cells of murine fibroblast L containing T7 promoter-dependent β-galactosidase gene and expressing the envelope glycoproteins of HIV-1_{NL43}. Native or 2 aa-truncated SDF-1α was added with a final concentration of 3 μg/ml, and sCD26 or mCD26 was added with a final concentration of 1 μg/ml. Results from independent triplicate experiments are indicated as level of infection calculated from the β-galactosidase activity observed in the absence of SDF-1α and recombinant soluble CD26 (100% of infection). Determined β-galactosidase activities in parent (A), CD26 (DPPIV$^+$) transfectant (B) and CD26 (DPPIV$^-$) transfectant (C) without SDF-1α and recombinant soluble CD26 were 0.149, 0.08 and 0.147 A_{595} increase/h, respectively. Addition of recombinant SDF-1α and recombinant soluble CD26 is indicated in the bottom line with abbreviations: —, not added; N, native SDF-1α; T, 2 aa-truncated SDF-1α; S, sCD26; M, mCD26. Asterisk and double asterisks mean $P<0.001$ and $P<0.005$ as compared with the result in the absence of SDF-1α and recombinant soluble CD26 (100% of infection) by Student's *t*-test, respectively.

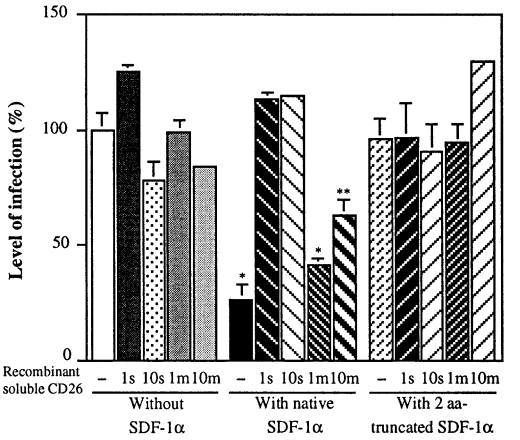

Fig. 2. Effect of DPPIV activity of sCD26 on inhibition of CXCR4-mediated infection of HIV-1_{NL43} by SDF-1α, monitored with β-galactosidase activity dependent on cell-cell fusion. 1×10^5 cells of murine fibroblast L transfected with pET-30b(+) containing β-galactosidase gene, expressing human CD4 and human CXCR4, were incubated with 1×10^5 cells of murine fibroblast L expressing T7 RNA polymerase and the envelope glycoproteins of HIV-1_{NL43}. Native or 2 aa-truncated SDF-1α was added at a final concentration of 3 μg/ml. Results from independent triplicate experiments are indicated as level of infection calculated from the β-galactosidase activity observed in the absence of SDF-1α and recombinant soluble CD26 (100% of infection). Determined β-galactosidase activity in mouse L cells without SDF-1α and recombinant soluble CD26 was 0.818 A_{595} increase/h. Addition of recombinant soluble CD26 is indicated as a final concentration with abbreviations: —, not added; 1s, 1 μg/ml of sCD26; 10s, 10 μg/ml of sCD26; 1m, 1 μg/ml of mCD26; 10m, 10 μg/ml of mCD26. Asterisk and double asterisks mean $P<0.001$ and $P<0.005$ as compared with the result in the absence of SDF-1α and recombinant soluble CD26 (100% of infection) by Student's *t*-test, respectively.

As the cleavage of SDF-1α by CD26/DPPIV was demonstrated, we attempted to determine whether the infectivity of T-tropic HIV-1_{NL43} was affected by the interaction between CD26 and SDF-1α by cell-cell fusion assay system using a Jurkat T cell line. This assay system made the experiment easy to investigate cell-entry step in HIV infection specifically because the color development dependent on β-galactosidase activity was observed only if cell-cell fusion between effector cells having the β-galactosidase gene under control of T7 promoter and responder cells having T7 RNA polymerase occurred. As shown in Fig. 1A, we confirmed that the infection by HIV was strongly inhibited in the presence of native SDF-1α (14.1% level of infection compared with the absense of either SDF-1α or recombinant soluble CD26). More significantly, the inhibitory activity of native SDF-1α was abolished by addition of sCD26 (57.7% level of infection) whereas the inhibitory activity still remained (26.2% level of infection) when mCD26 was added (Fig. 1A). In marked contrast, the infection was not inhibited by addition of 2 aa-truncated SDF-1α, or addition of either sCD26 or mCD26 alone (Fig. 1A). In this regard, the importance of N-terminus of SDF-1α has been shown to block infection by T-tropic HIV strains, and also N-terminal truncated SDF-1α failed to block the infection [21,22,29]. These results suggested that native SDF-1α appears to lose the inhibitory activity by CD26-mediated cleavage. In Fig. 1A we can see a slight disappearance of the

inhibitory activity in native SDF-1α incubated with mCD26. It should be noted that native SDF-1α incubated with mCD26 was also cleaved when an excess amount of mCD26 (50 μg per 5 μg of SDF-1α) was added and in vitro cleavage was performed overnight, although the DPPIV activity of mCD26 was undetectable with colorimetric assay. In rat DPPIV, Ogata et al. [30] described that it is essential to preserve not only the catalytic site of Ser^{631} (corresponding to Ser^{630} of human DPPIV) but also Gly^{629}, Tyr^{632} and Gly^{633} (Gly^{628}, Tyr^{631} and Gly^{632} in human DPPIV) for proteolytic activity. Since the recombinant soluble CD26 lacking DPPIV activity was created by a single substitution of Ala for the putative catalytic Ser residue at position 630 [27], this mutation may not be sufficient to lose the DPPIV activity completely.

Next we determined whether the cell surface CD26 molecule interacts with SDF-1α and this interaction affects the infectivity of HIV. For this purpose, we utilized wild-type CD26 ($DPPIV^+$) and mutant CD26 ($DPPIV^-$) transfectants of Jurkat cell line as we previously established [27]. As shown in Fig. 1B, when the transfectants expressing wild-type CD26 ($DPPIV^+$) were used, native SDF-1α did not inhibit HIV infection (82.5% level of infection). Moreover, the inhibitory activity of HIV infection by SDF-1α was not affected by the presence of sCD26 or mCD26. However, in mutant CD26 ($DPPIV^-$) transfectant, SDF-1α inhibited the HIV infection (26.5% level of infection; Fig. 1C). In these cells, similar to the results observed in parent Jurkat cells, the inhibitory activity of SDF-1α disappeared in the presence of sCD26 (95.2% level of infection), and the modest inhibitory activity of HIV infection by SDF-1α was observed in the presence of mCD26 (61.2% level of infection; Fig. 1C). The addition of 2 aa-truncated SDF-1α consistently did not inhibit the HIV infection in either of the Jurkat CD26-transfectants regardless of the presense of sCD26 or mCD26 (80–170% level of infection; Fig. 1B,C). These results indicate that the presence of CD26/DPPIV on cell surface causes loss of inhibitory activity of SDF-1α against HIV. Interestingly, the transfectant expressing wild-type CD26 ($DPPIV^+$) was relatively resistant against HIV infection in the absence of SDF-1α and sCD26, as compared with the parent Jurkat T cell line (50% level of infection compared to parent cells) which we also observed previously [27]. It is conceivable that an interaction of CD26 with other cell surface molecules or other factors may affect the infectivity of HIV. Further studies are now in progress to define why cells expressing CD26 are resistant to HIV infection.

To determine more specifically the influence of the interaction between SDF-1α and sCD26 upon HIV infection, we next performed a cell-cell fusion assay using murine fibroblast L cells expressing human CD4 and human CXCR4 as the responder cells instead of the Jurkat T cell line. As shown in Fig. 2, it was confirmed that native SDF-1α strongly inhibited HIV infection (26.3% level of infection), the inhibitory activity disappeared in the presence of sCD26 (113% level of infection), and the 2 aa-truncated form of SDF-1α was unable to inhibit the HIV infection (95.7% level of infection). Addition of a 10-fold amount of sCD26 alone (final concentration of 10 μg/ml) did not affect the HIV infection, indicating that soluble CD26 might not interact with HIV envelope (Fig. 2). These findings further emphasize that the interaction between SDF-1α and CD26, whether it was presented on cell surface or as a soluble form, occurred through proteolytic cleavage since the anti-HIV activity of native SDF-1α was lost only when the DPPIV activity of CD26 was present in either mouse L cells or the Jurkat T cell line.

Since chemokines play a critical role in T cell migration and in vivo migrating T cells expressed a high level of CD26, we next investigated the effect of the interaction between sCD26 and SDF-1α on T cell migration. As shown in Table 1, native SDF-1α had a chemotactic activity for T cells in agreement with previous studies [21,22,29]. It is noteworthy that the chemotactic activity of SDF-1α was abolished by addition of sCD26 whereas the chemotactic activity of SDF-1α was not affected in the presence of mCD26 (Table 1). These results indicated that the N-terminal cleavage of SDF-1α by sCD26 caused disappearance of the function not only to block HIV infection but also to induce T cell chemotaxis. In the light of previous observations that the N-terminal 2 aa residues of SDF-1α are essential for cell migration and that receptor signalling of 2 aa-truncated SDF-1α is not detected although the binding activity was retained [29], our findings indicate that CD26/DPPIV physiologically modulates chemotactic activity of SDF-1α via cleavage of the N-terminus essential for induction of chemotaxis.

The cleavage of SDF-1α by CD26 found in this study is easily considered to occur in vivo. T-tropic HIVs are known to be rampant in HIV-infected individuals at a late stage of the disease. Considering the fact that soluble CD26 with DPPIV activity is present in human plasma [31] and that CD26 molecules play an important role in memory T cell activation and function [5,6,26], there is a possibility that CD26 counteracts the protective measure in human body against T-tropic HIV, including SDF-1α, and would even facilitate HIV dissemination. However, for M-tropic HIV which is generally found in an early stage of the disease, RANTES was recently reported to retain or rather enhance its anti-HIV activity via an interaction with the receptor CCR5 after cleavage by CD26/DPPIV, though the cleaved RANTES had less affinity with another receptor, CCR1 [24,32]. So far it has been described that several chemokines other than SDF-1 and RANTES contain the conserved cleavage site of CD26/DPPIV. Therefore, it is expected that an in vivo general modulation of various chemokines is induced by cell surface CD26 as well as soluble form CD26 in plasma.

In summary, we showed that a chemokine, SDF-1α, is a substrate for CD26/DPPIV, and interaction between both membrane and soluble forms of CD26/DPPIV and SDF-1α caused the disappearance of the inhibitory activity against HIV infection and migratory activity for T cells. These results indicated that CD26/DPPIV is a potent intrinsic modulatory factor of protective as well as inflammatory responses through interaction with chemokines.

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