Enhanced anti-HIV-1 activity of CC-chemokine LD78β, a non-allelic variant of MIP-1α/LD78α

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Abstract We compared the anti-HIV-1 activity of CC-chemokine LD78β with that of MIP-1α, another CC-chemokine which shows 94% sequence homology with LD78β. Despite its close similarity to MIP-1α, the anti-HIV-1 activity of LD78β appeared to be nearly 10 times higher than that of MIP-1α. Mutagenesis of MIP-1α showed that the N-terminal additional tetrapeptide, which was present in LD78β and absent in MIP-1α, is responsible for enhanced anti-HIV-1 activity. The N-terminal structure-function relationship of LD78β described here will be of value in understanding the chemokine-receptor interactions and designing anti-HIV-1 compounds based on LD78β.

Key words: Human immunodeficiency virus type 1; Chemokine; Macrophage inflammatory peptide 1α; LD78β; CD26/dipeptidyl peptidase IV

1. Introduction

Chemokines constitute a large family of small chemotactic cytokines of 60–80 amino acid residues. There are two major subfamilies of chemokines, CC-chemokines and CXC-chemokines, which differ in their spacing of the first two cysteine residues [1,2]. Two exceptions to the CC/CXC rule have been reported: one is a C-chemokine, lymphotactin, and another is a CX_3C-chemokine, fractalkine [1,2]. Recently, some of the receptors of CC-chemokines and CXC-chemokines were proved to serve as essential coreceptors for cellular entry of human immunodeficiency virus type 1 (HIV-1) [3–9]. Coreceptor usage by HIV-1 is strain-specific. For instance, macrophage tropic/non-syncytium-inducing strains use CC-chemokine receptor 5 (CCR5) for their entry into cells [3,5–8]. On the other hand, CXC-chemokine receptor 4 (CXCR4) serves as a coreceptor for T-cell line tropic/syncytium-inducing strains [4,9].

MIP-1α (macrophage inflammatory peptide 1α), also referred to as LD78α, is one of the natural ligands of CCR5, and is able to induce chemotaxis of many mature leukocyte types [1,2]. MIP-1α strongly inhibits proliferation of hematopoietic stem cells [10,11] and moderately suppresses in vitro replication of macrophage tropic strains of HIV-1, which use CCR5 as a coreceptor [12]. LD78β, a non-allelic variant of MIP-1α, shows more than 94% sequence homology with MIP-1α [13,14]. Despite its close similarity to MIP-1α, biological activities of LD78β on HIV-1 infection have not been fully clarified yet. In this report, we demonstrate that recombinant LD78β expressed in mammalian cells by Sendai virus (SeV) vector [15,16,17] is able to suppress replication of macrophage tropic strains of HIV-1 10 times more efficiently than recombinant MIP-1α. The N-terminal tetrapeptide of LD78β, which is absent in MIP-1α, appears to be responsible for the elevated anti-HIV-1 activity.

2. Materials and methods

2.1. Cells and virus

Human peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers (Japanese Red Cross Central Blood Center) were isolated by Ficoll/Hypaque gradient centrifugation. CD4+ cells were purified from the PBMC with magnetic beads coated with anti-CD4 antibody as described previously [18]. Monkey kidney CV1 cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). HIV-1 strain SF162 was propagated in phytohemagglutinin (PHA)-stimulated PBMC which were maintained in RPMI 1640 containing 32 U/ml human interleukin-2 (IL-2, Boehringer Mannheim, Germany) and 10% FBS.

2.2. Generation of recombinant SeV

MIP-1α cDNA was cloned from PHA and 12-myristate 13-acetate-stimulated PBMC by nested RT-PCR. LD78β and its mutant genes were generated by site-directed mutagenesis by using two or three successive PCRs using MIP-1α cDNA as a template. All PCRs were performed with ExTaq DNA polymerase (Takara, Kyoto, Japan), and all the PCR products were verified for their sequence authenticity. Recombinant SeV carrying MIP-1α, LD78β and their derivatives were generated according to the method described previously [15–17,19].

2.3. Production of human MIP-1α, LD78β and their derivatives

CV1 cells were infected with recombinant SeV expressing MIP-1α, LD78β, or their derivatives at 10 plaque-forming units (PFU) per cell. Seventy-two hours after infection, serum-free culture supernatants were harvested and centrifuged at $48{,}000 \times g$ for 2 h at 4°C. The amount of MIP-1α and its derivatives in the culture supernatant was determined by Human MIP-1α Immunoassay Kit (R&D, USA).

2.4. Western blotting

The culture supernatant of infected cells was electrophoresed in 15% polyacrylamide gels (Mini-Protean II Ready Gels J, Bio-Rad). The proteins in the gels were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 100 mA for 1 h and probed with anti-MIP-1α antibody (Genzyme, USA).

2.5. CD26-mediated cleavage of LD78β

Murine fibroblast L cells expressing CD26 [20] were infected with recombinant SeV expressing LD78β at 30 PFU per cell and maintained in serum-free Dulbecco's modified Eagle's medium (DMEM)

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for 72 h. LD78β in the culture supernatants was analyzed by Western blotting and N-terminal peptide sequencing (Takara, Kyoto, Japan).

2.6. Anti-HIV-1 assay

PBMC obtained from healthy donors (1.5×10^6/ml) were stimulated with PHA for 3 days and exposed to the macrophage tropic HIV-1 strain SF162 equivalent to 12 ng of p24 antigen for 2 h at 37°C. The cells were washed three times and then maintained in the medium described above in the presence of indicated concentrations of LD78-derived chemokines. The culture supernatants were harvested 3 days after infection and assayed for the levels of p24 core antigen (Abbott, Wiesbaden-Delkenheim, Germany).

2.7. Competitive binding assay of LD78β

Receptor binding activity of LD78-derived chemokines was assayed by competitive binding of these chemokines with ^{125}I-labeled MIP-1α to purified CD4+ cells. We modified a protocol originally described by Haribabu et al. [21]. Briefly, 2×10^6 PHA-stimulated CD4+ cells were incubated in 200 μl of DMEM containing 25 mM HEPES, 1% BSA, 1 nM of ^{125}I-labeled MIP-1α (0.25 mCi, NEN Life Science Products, Boston, MA, USA) and increasing concentrations of unlabeled LD78-derived chemokines. After incubation for 2 h, the cells were washed twice with DMEM containing 25 mM HEPES and 1% BSA and cell-bound radioactivity was counted on a 1470 WIZARD Gamma Counter (Wallac, USA). Inhibition of binding of ^{125}I-labeled MIP-1α was determined by the formula (An−Bn)100%/An, where An is the cpm bound in the absence of any competitors; Bn is the cpm bound in the presence of indicated concentration of competitors.

3. Results

3.1. Expression of MIP-1α and LD78β by a SeV vector

Four to five μg/ml of MIP-1α was detected in the culture supernatant of CV1 cells infected with the recombinant SeV carrying the human MIP-1α gene. A similar level of LD78β was detected in the culture supernatant of CV1 cells infected with the SeV carrying the LD78β gene (Fig. 1A). The N-terminal sequence analysis of recombinant MIP-1α revealed a

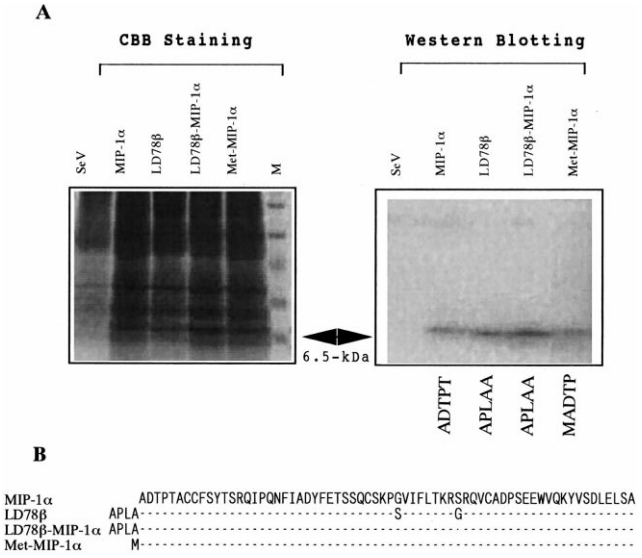

Fig. 1. Expression of MIP-1α, LD78β, LD78β-MIP-1α, and Met-MIP-1α. A: Proteins in culture supernatants of CV-1 cells infected with SeV carrying indicated chemokine genes or the wild type SeV (SeV) were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-MIP-1α antibody. M indicates protein size markers. The position of a 6.5-kDa marker is shown by the arrow. The N-terminal amino acid sequence of each protein band is shown. B: Alignment of amino acid sequences of MIP-1α, LD78β, LD78β-MIP-1α, and Met-MIP-1α. Dashes indicate sequence identity.

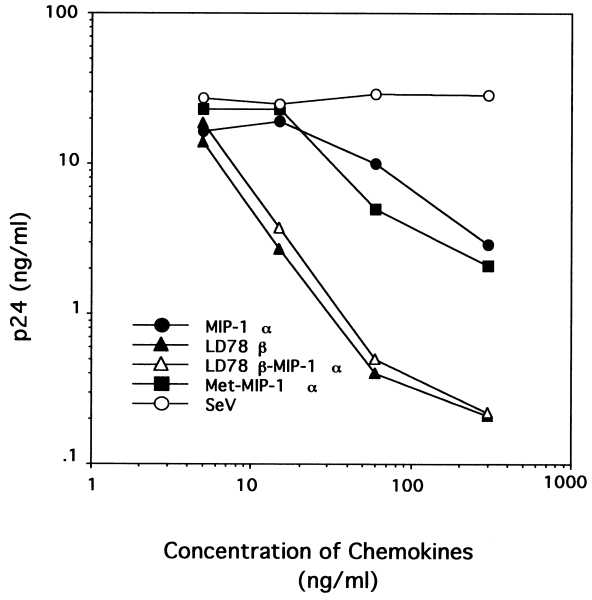

Fig. 2. Anti-HIV-1 activity of MIP-1α (closed circles), LD78β (closed triangles), LD78β-MIP-1α (open triangles), and Met-MIP-1α (closed squares). Culture supernatants of CV-1 cells infected with SeV carrying indicated chemokine genes or the wild type SeV (open circles) were serially diluted and assayed for their anti-HIV-1 activity as described in Section 2. Data shown are representative of three independent experiments with similar results.

pentapeptide, ADTPT, in its N-terminus (Fig. 1A,B), instead of the tetrapeptide ASLA which was previously suggested to be present at the N-terminus of mature MIP-1α [13]. In contrast, the N-terminal tetrapeptide APLA was fully retained in recombinant LD78β (Fig. 1A,B). Therefore, mature MIP-1α appears to be composed of 66 amino acids, whereas mature LD78β consists of 70 amino acids.

3.2. Anti-HIV-1 and receptor binding activities of MIP-1α and LD78β

Recombinant MIP-1α and LD78β were assayed for their anti-HIV-1 activity. As shown in Fig. 2, MIP-1α suppressed 90% of p24 production of HIV-1 macrophage tropic strain SF162 in PBMC culture at a concentration of 300 ng/ml. In contrast, LD78β suppressed more than 99% of p24 production at a concentration of 300 ng/ml and suppressed 90% of p24 production even at a concentration of 15 ng/ml. These data clearly indicate that LD78β is a more potent anti-HIV-1 suppressor than MIP-1α.

To evaluate binding affinity of MIP-1α and LD78β to their receptors, we performed competitive binding assays of MIP-1α and LD78β to CD4+ cells with ^{125}I-labeled MIP-1α (Fig. 3). 70 ng/ml of MIP-1α was required for 50% inhibition of radiolabeled MIP-1α binding, while 20 ng/ml of LD78β was sufficient for 50% inhibition, indicating that LD78β has higher receptor binding affinity than MIP-1α.

3.3. The N-terminal tetrapeptide APLA is responsible for enhanced anti-HIV-1 and receptor binding activities of LD78β

Except for the N-terminal tetrapeptide, there are two amino acid differences between mature MIP-1α and LD78β (Fig. 1B). To test whether the N-terminal tetrapeptide APLA is

responsible for the elevated anti-HIV-1 and receptor binding activity of LD78β, we constructed chimeric MIP-1α carrying the N-terminal tetrapeptide APLA (LD78β-MIP-1α). We also generated mutant MIP-1α carrying an additional methionine residue at the N-terminus (Met-MIPα). The LD78β-MIP-1α and Met-MIP-1α were expressed and secreted as efficiently as the wild type MIP-1α and LD78β into culture supernatant of CV-1 cells infected with recombinant SeV carrying the respective mutant MIP-1α genes (Fig. 1A). The N-terminal amino acid sequence analysis confirmed the presence of the tetrapeptide and a methionine residue in mature LD78β-MIP-1α and Met-MIP-1α, respectively.

As shown in Fig. 2, the chimeric LD78β-MIP-1α suppressed HIV-1 replication as efficiently as did LD78β. LD78β-MIP-1α also showed elevated binding affinity to CD4+ cells (Fig. 3). In contrast, anti-HIV-1 and receptor binding activity of Met-MIP-1α was the same as for MIP-1α (Figs. 2 and 3). Therefore, the N-terminal tetrapeptide but not a single additional amino acid residue is responsible for the elevated anti-HIV-1 and receptor binding activity of LD78β.

3.4. Effect of CD26/dipeptidyl peptidase IV-mediated cleavage of LD78β

We and others have shown that CD26/dipeptidyl peptidase IV was able to process several chemokines such as SDF-1α and SDF-1β [20], RANTES [22,23], MCP-2 [24], and MDC [25]. CD26 cleaves the first two amino acids from the polypeptide carrying proline or alanine at the second position. Since the N-terminal tetrapeptide of LD78β was APLA, a possible substrate for CD26, we tested whether CD26-mediated cleavage of LD78β affects its anti-HIV-1 and receptor binding activity. For this purpose, L cells expressing human CD26 [20] were infected with the recombinant SeV expressing LD78β, and the N-terminal sequence and the anti-HIV-1 and

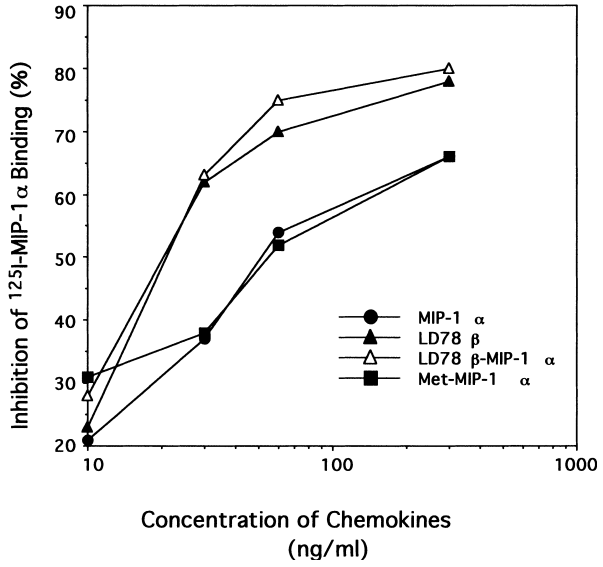

Fig. 3. Receptor binding activity of MIP-1α (closed circles), LD78β (closed triangles), LD78β-MIP-1α (open triangles), and Met-MIP-1α (closed squares). Culture supernatants of CV-1 cells infected with SeV carrying indicated chemokine genes were serially diluted and assayed for their receptor binding activity as described in Section 2. Data shown are representative of three independent experiments with similar results.

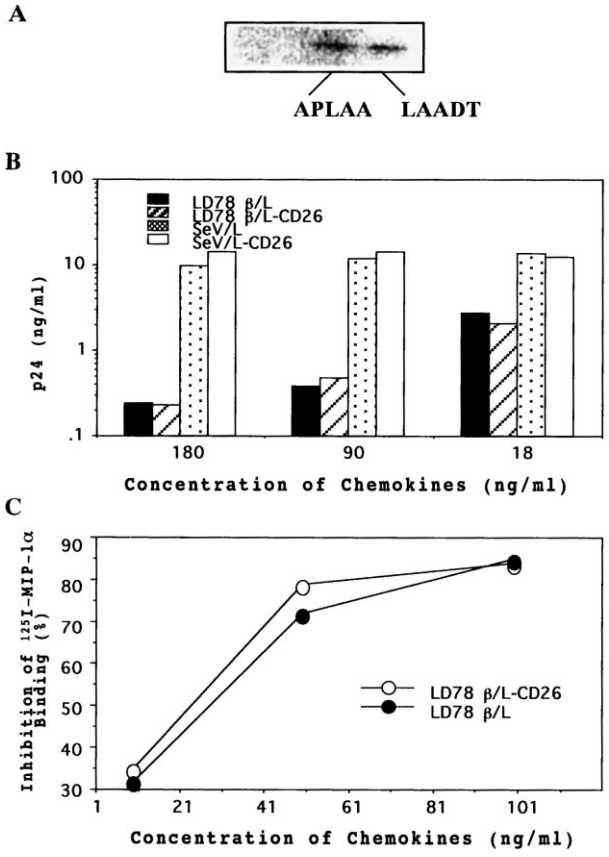

Fig. 4. Effects of CD26-mediated cleavage of LD78β on its biological activities. A: Proteins in culture supernatants of L cells expressing CD26 (L-CD26) or control L cells (L) infected with SeV carrying the LD78β gene (LD78β) or the wild type SeV (SeV) were analyzed by Western blotting using anti-MIP-1α antibody. The N-terminal amino acid sequence of each protein band is shown. B: Culture supernatants of L cells expressing CD26 (L-CD26) or control L cells (L) infected with SeV carrying the LD78β gene (LD78β) or the wild type SeV (SeV) were serially diluted and assayed for their anti-HIV-1 activity. The levels of p24 antigen production 3 days after infection are shown. C: Culture supernatants of L cells expressing CD26 (open circles) or control L cells (closed circles) infected with SeV carrying the LD78β gene were serially diluted and assayed for their receptor binding activity.

receptor binding activities of LD78β produced in the presence of CD26 were analyzed. Since the N-terminal amino acid sequence of LD78β secreted from L cells expressing CD26 was LAADTP (Fig. 4A), the N-terminal dipeptide AP was in fact removed by CD26. On the other hand, the dipeptide was fully retained in the LD78β secreted from control L cells without CD26 expression (Fig. 4A), indicating that LD78β is a substrate of CD26. However, as shown in Fig. 4B and 4C, LD78β produced in the presence of CD26 exhibited similar levels of anti-HIV-1 and receptor binding activities to those of LD78β produced in the absence of CD26, showing that the N-terminal dipeptide AP is not responsible for the elevated biological activity of LD78β.

4. Discussion

In this paper, we demonstrated that LD78β, a non-allelic variant of MIP-1α, has higher anti-HIV-1 and receptor binding activities than MIP-1α. The N-terminal tetrapeptide APLA of LD78β which is absent in MIP-1α appeared to be

responsible for the elevated anti-HIV-1 and receptor binding activities. However, CD26-mediated removal of the N-terminal dipeptide AP from LD78β did not alter its anti-HIV-1 and receptor binding activities (Fig. 4), suggesting that dipeptide LA next to the N-terminal dipeptide AP plays a critical role in elevating biological activity of LD78β. An extensive mutagenic study of the N-terminal portion of LD78β is now in progress to prove this hypothesis.

It is now clear that CD26-mediated cleavage of the N-terminal dipeptide cause different impacts on different chemokines. Anti-HIV-1 and chemotactic activities of SDF-1α and SDF-1β are abolished by CD26-mediated cleavage [20]. In contrast, RANTES lacking the N-terminal dipeptide loses its ability to interact with CCR1 while it retains CCR5 interaction and thus could suppress replication of macrophage tropic strains of HIV-1 [22,23]. The present study showed that LD78β, another natural ligand of CCR5, also retained its anti-HIV-1 and receptor binding activities after CD26-mediated cleavage. In the human body, CD26 would facilitate replication of HIV-1 with a T-cell line tropic phenotype, while it has no effect on HIV-1 with a macrophage tropic phenotype. It is possible that the level of CD26 expression may be one of the key determinants for phenotypic shift of macrophage tropic HIV-1 to T-cell-line tropic HIV-1.

Recent studies showed that the levels of the production of CC-chemokine including MIP-1α may play important roles in HIV-1 transmission and disease progression to AIDS [26–30]. Since copy numbers of the LD78β gene were reported to vary among individuals [13,14], it would be important to determine the LD78β copy numbers of HIV-1-infected individuals with different rates of disease progression as well as individuals who were exposed to HIV-1 but still remain uninfected.

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