Chemically Synthesized SDF-1α Analogue, N33A, Is a Potent Chemotactic Agent for CXCR4/Fusin/LESTR-expressing Human Leukocytes*

(Received for publication, June 26, 1997, and in revised form, July 30, 1997)

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Stromal cell-derived factor (SDF) 1 is a potent chemoattractant for leukocytes through activation of the receptor CXCR4/Fusin/LESTR, which is a fusion co-factor for the entry of T lymphocytotropic human immunodeficiency virus type 1 (HIV-1). This CXCR4-mediated HIV-1 fusion can be inhibited by SDF-1. Because of its importance in the study of immunity and AIDS, large scale production of SDF-1 is desirable. In addition to recombinant technology, chemical synthesis provides means by which biologically active proteins can be produced not only in large quantity but also with a variety of designed modifications. In this study, we investigated the binding and function of an SDF-1α analogue, N33A, synthesized by a newly developed native chemical ligation approach. Radioiodinated N33A showed high affinity binding to human monocytes, T lymphocytes, as well as neutrophils, and competed equally well with native recombinant SDF-1α for binding sites on leukocytes. N33A also showed equally potent chemoattractant activity as native recombinant SDF-1α for human leukocytes. Further study with CXCR4/Fusin/LESTR transfected HEK 293 cells showed that N33A binds and induces directional migration of these cells in vitro. These results demonstrate that the chemically synthesized SDF-1α analogue, N33A, which can be produced rapidly in large quantity, possesses the same capacity as native SDF-1α to activate CXCR4-expressing cells and will provide a valuable agent for research on the host immune response and AIDS.

Stromal cell-derived factor (SDF) 1 has been reported to be a primordial chemokine of the CXC subfamily and has multiple biological activities on a variety of cell types (1–4). SDF-1 was initially isolated as a T lymphocyte chemoattractant and was found to be active also on monocytes but not on neutrophils (1, 3). However, its activity on neutrophils was subsequently shown by using Ca2+-mobilization experiments (4). The SDF-1 gene is located on chromosome 10 (5), while the genes for other known CXC chemokines are located chromosome 4 (6, 7). SDF-1 is well conserved with only a single amino acid substitution between human and murine molecules (1, 2). SDF-1α and SDF-1β were believed to be the result of differential splicing of a single gene, with SDF-1α missing four amino acids in the carboxyl terminus. The CXCR4/Fusin/LESTR has been identified as one of the functional receptors for SDF-1 (3, 4), a member of the seven transmembrane spanning receptor superfamily (8, 9). CXCR4 was initially cloned as an orphan receptor (10–12) and was later identified as a fusion co-factor for the entry of HIV-1 of the T lymphotrophic strain (13). In addition to its activities on human leukocytes, SDF-1 has been shown to inhibit the fusion and replication of T lymphotropic HIV-1 in host cells bearing CD4 and CXCR4 (3, 4). Therefore, SDF-1 plays a pivotal role in host immune system and its defense against infection.

Rapid production of cytokines and chemokines is essential for structure-function studies and design of molecules with agonist or antagonist activities. The turbocharged peptide synthesis technology (14) and chemical ligation of peptide segments (15) permit the synthesis of chemokines in large quantity. This approach was therefore utilized to synthesize SDF-1α based on its great utility in studies of immune responses and infectious diseases. However, the thioacid peptide (SDF-1α(1–33)-cCOSH) could not be readily generated on the standard Fmoc-based titanosilica resin (16). The SDF-1α(1–33) residue at the ligation site in SDF-1α. Instead, alanine was coupled to the α-thiocarboxylate resin, then the mutant SDF-1α(1–33, N33A) was synthesized. In this study, we report that this SDF-1α analogue, N33A, displays full biological activity through the activation of the receptor CXCR4. This analogue, which can be rapidly produced in large quantity in the absence of contaminating peptides, will prove to be an important tool in the study of host immunity and AIDS.

MATERIALS AND METHODS

Chemokines—Human recombinant SDF-1α was purchased from PeproTech Inc. (Rocky Hill, NJ). Radioiodination of the SDF-1α and the analogue N33A was performed by a lactoperoxidase-labeling procedure. The radioactive ligands were further purified by reversed phase HPLC. The specific activity of the radiiodinated chemokines was 2200 Ci/mmol.

Chemical Synthesis of SDF-1α Analogue, N33A—Boc protected amino acids were obtained from the following sources: AnaSpec (San Jose, CA); Bachem (Philadelphia, PA), NovaBiochem (San Diego, CA), and Peptides International (Louisville, KY). Peptides were synthesized on a modified ABI430A instrument using in situ neutralization Boc chemistry protocols (17). C-terminal segments were prepared on –OCH2Pam resins (ABI, Foster City, CA). N-terminal segments were prepared on α-thiocarboxylate resin (16). Standard HF cleavage protocols were employed following N-terminal Boc removal and drying of the

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‡ The abbreviations used are: SDF, stromal cell-derived factor; HIV-1, human immunodeficiency virus type 1; HPLC, high pressure liquid chromatography; Boc, t-butoxycarbonyl; CI, chemotaxis index; rh, recombinant human.
HCl (Fluka), 0.1 M Tris (Fluka), pH 8.6, at room temperature in the
atmosphere at 40 °C for 1 h. After complete reduction, the mixture was
monitored by HPLC and was typically complete within 24 h. Ligation
was followed by HPLC purification and lyophilization. After purifica-
tion, the full-length peptide was reduced at 1 mg/ml in 8M urea (Fluka),
0.1 M Tris (Fluka), 5.37 mM EDTA ((Fluka), pH 8.6, in the presence of
33 mM thiophenol (Fluka). Reduction occurs under a nitrogen
folding was complete after stirring overnight and was
concentration in 6 M guanidine, 0.1 M Tris, pH 8.6. Over a period of 2 days, 4 liters of 2 M urea, 0.1 M Tris, 1 mM EDTA, 3 mM 2-mercaptoethanol, 1.3 mM oxidized glutathione, pH
8.6. Over a period of 2 days, 4 liters of 2 M urea, 0.1 M Tris, pH 8.6, was
pumped into the vessel containing the dialysis bag (18). After ligation,
the full-length peptide was oxidized at 1 mg/ml in 2 M guanidine
HCl (Fluka), 0.1 M Tris (Fluka), pH 8.6, at room temperature in the
presence of air. Folding was complete after stirring overnight and was
monitored by HPLC and mass spectrometry.

Cells and Chemotaxis Assays—Human peripheral blood leukocytes
were isolated from normal donors (National Institutes of Health Clinical
Center Transfusion Department, Bethesda, MD) according to the
established protocols in this laboratory for cultivation of monocytes
(purity >90%) (19), T lymphocytes (purity, >95% CD3+ CD8+) (20) and
neutrophils (purity, >98%) (19). The chemokine receptor, CXCR4/fusin/
LESTR cDNA was isolated in this laboratory and was stably trans-
formed into human kidney embryonic epithelial 293 cells (CXCR4/293
cells) as described previously (21). Leukocyte migration was evaluated using a 48-well microchamber (Neuroprobe, Cabin John, MD) technique as described previously (19, 20, 22). The migration of CXCR4/293 cells was also assessed by the
48-well microchamber technique with the polycarbonate filters (10-μm
core size) (21) precoated with collagen type I (Collaborative Biomedical
Products, Bedford, MA). The results are expressed as the the chemokine/binding in the presence of medium alone) × 100 (Eq. 1)

RESULTS AND DISCUSSION

We first examined whether the SDF-1α analogue N33A was able to bind and activate human peripheral blood monocytes and T cells. Fig. 1 shows that 125I-N33A specifically bound to human peripheral blood monocytes (Fig. 1A) and T lymphocytes (Fig. 1B) with high affinity (1.8 and 1.4 nM for monocytes and T cells, respectively). This level of binding to monocytes and T cells by 125I-N33A was comparable to that of 125I-rhSDF-1α as examined in parallel experiments (data not shown). Unlabeled N33A displaced 125I-rhSDF-1α binding to both monocytes and T lymphocytes (Fig. 1, C and D), and likewise, the binding of 125I-N33A to these cell types was also displaced by both unlabeled N33A and rhSDF-1α (not shown). Consistent and considerable migration of monocytes and T cells was induced by N33A (Fig. 2). The potency and efficacy of N33A in the induction of mononuclear cell migration was comparable with rhSDF-1α, suggesting that chemically synthesized N33A retains the tertiary structure and function as well as rhSDF-1α.

The effect of SDF-1 on neutrophils is controversial (1, 3, 4). While some investigators failed to detect chemotactic activity of SDF-1 for neutrophils (1), others were able to induce significant Ca2+ mobilization in neutrophils at physiologically relevant concentrations of SDF-1 (4). In an effort to clarify the activity of SDF-1 on neutrophils, we tested the binding and function of
N33A on neutrophils in comparison to rhSDF-1α. As shown in Fig. 3, human peripheral blood neutrophils expressed a substantial number of specific binding sites for both N33A (Fig. 3A) and native SDF-1α (Fig. 3B). The binding is of high affinity with estimated $K_d$ values of about 5 nM. N33A and rhSDF-1α competed equally well for each other’s binding as shown by the displacement curve (Fig. 3C and data not shown). Neutrophils also migrated in response to both N33A and rhSDF-1α, indicating that neutrophils are indeed among the target cell types for SDF-1.

To further confirm that N33A utilizes CXCR4 as its functional receptor, HEK293 cells stably expressing CXCR4 (CXCR4/293 cells) were employed. Wild type HEK 293 cells exhibited a low level of specific binding (about 200 binding sites/cell) for both N33A and native SDF-1α. Both N33A and native SDF-1α also induced a weak but significant directional migration of wild type HEK293 cells ($CI^{2} = 2.1 ± 0.2$, at 120 nM ligand concentration). This is in agreement with the notion that a great variety of nonhematopoietic cells express CXCR4 mRNA. However, HEK293 cells overexpressing CXCR4 expressed markedly increased number of specific binding sites for radiolabeled N33A and rhSDF-1α (Fig. 4, A and B). N33A and rhSDF-1α mutually competed for binding to CXCR4/293 cells (Fig. 4, C and D). Both N33A and rhSDF-1α were able to induce a remarkable directional migration of CXCR4/293 cells (Fig. 5) with similar potency and efficacy. HEK293 cells transfected with known chemokine receptors, including CXCR1, CXCR2, and CCR1–5, did not show any increased binding or activation by N33A or rhSDF-1α over background levels, whereas these cells specifically bound and migrated in response to their ligands (data not shown). These results demonstrate that N33A, like rhSDF-1α, uses CXCR4 as a functional receptor.

Chemokines are important mediators that participate in a variety of pathophysiological conditions including inflammation, infection, tissue injury and repair, immune responses, as well as malignancy (6, 7). Recently members of chemokine receptor family have been identified to be fusion co-factors for HIV-1 entry into host cells. Some chemokines were able to inhibit HIV-1 entry through competitive occupancy of the relevant receptors such as CCR5 or CXCR4/Fusin/LESTR which mediate the fusion of either monocytotropic or T lymphocytotropic viruses, respectively, with CD4+ host cells (2, 3, 13, 23–27). SDF-1 has recently been identified as the ligand for CXCR4 and was able to inhibit the cell fusion mediated by the envelope protein of the T lymphocytotropic HIV-1 strain (23–28).

SDF-1 is a member of the CXC chemokine subfamily and was first identified as a molecule possessing pre-B cell-stimulatory activity (29, 30). It was later described as a chemoattractant for resting T lymphocytes and monocytes (1). As a CXC chemokine, SDF-1 has several unique features in comparison to other...
members of the same family. SDF-1 mRNA is expressed constitutively in virtually every tissue including heart, liver, lung, brain, muscle, spleen, and kidney (28–30). Expression of SDF-1 gene is not affected by proinflammatory stimuli (31), in contrast to most other chemokines which are mainly expressed in response to proinflammatory cytokines and are believed to regulate the recruitment and activation of mature leukocytes at inflammatory foci (6, 7). In the absence of inflammation, blood monocytes constantly replace mononuclear phagocytes in the tissue, sustaining a stable level by extravasation from bloodstream and undergoing differentiation into macrophages. SDF-1 displays a tissue distribution that is considered appropriate for function in lymphocyte recirculation, in basal recruitment of monocytes and in normal replenishment and turnover of tissue mononuclear phagocytes (1, 30–32). However, neutrophils do not normally infiltrate organs or tissues even though these cells express binding sites for and migrate in response to SDF-1α in vitro, as further demonstrated in the current study. Thus, the role for SDF-1 as a primordial chemokine regulating primarily the tissue distribution of leukocytes needs further investigation.

SDF-1 also has several essential functions in development (2). Mice lacking SDF-1 due to homologous recombination died perinatally and although the numbers of B cell progenitors in mutant embryos were severely reduced in fetal liver and bone marrow, myeloid progenitors were reduced only in the bone marrow not in the fetal liver, indicating that SDF-1 is responsible for B cell lymphopoiesis and bone marrow myelopoiesis. In addition, mice deprived of SDF-1 gene had a cardiac ventricular septal defect (2).

The SDF-1α analogue N33A was chemically synthesized and was shown in this study to be equally as potent as native SDF-1α in binding and activating human leukocytes as well as CXCR4-transfected HEK293 cells. N33A has also been shown to induce human B lymphocyte migration and to effectively inhibit infection of PM-1 cells by HIV-1(LAV).2 The preparation of analogues is greatly facilitated by molecular chemical synthesis, in which proteins can be produced either singly or by combinational methods. The analogues could include a full range of genetically encoded amino acids as well as unnatural backbone structures and unclonable residues such as D-amino acids, fluorescent or nuclear magnetic resonance-sensitive nuclei. The activities of analogues can also be tuned by fast cycles of synthesis-design-assay-resynthesis (33). The validity of this approach is demonstrated by the fact that it yields functional molecules such as the SDF-1α analogue N33A, a biological

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2 M. A. Siani, D. A. Thompson, L. E. Canne, G. M. Figliozzi, S. B. H. Kent, R. Simon, J. Cyster, P. E. Kennedy, E. D. Smith, and E. A. Berger, unpublished observation.
contamination-free agonist of SDF-1 that can be utilized in studies of the immune system and host defense against AIDS.

Acknowledgment—We thank Kathleen Bengali and Nancy Dunlop for technical support, and Cheryl Fogle and Teresa Covell for secretarial assistance. The critical review of this manuscript by Dr. Joost J. Oppenheim is greatly appreciated.

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