Increased Level of Stromal Cell-Derived Factor-1 mRNA in Peripheral Blood Mononuclear Cells from Children with AIDS-related Lymphoma

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ABSTRACT

A common polymorphism in the 3′ untranslated region of the stromal cell-derived factor 1 (also called pre-B-cell-stimulating factor) β gene transcript, termed SDF1–3′A, has been associated with an increased risk of non-Hodgkin’s lymphoma (NHL) in HIV-infected, but not in uninfected, individuals. Because the gene variation is located within the 3′ untranslated region, the SDF1–3′A may influence the abundance of SDF-1 mRNA, possibly up-regulating the chemokine expression especially in the presence of HIV-1. In the current study, we investigated the levels of SDF-1 mRNA in peripheral blood mononuclear cells and HIV-1 viral load in 84 HIV-infected children (0.7 to 18 years of age; median, 5.8), including 12 children who developed NHL during their illnesses (AIDS-NHL group; 8 with SDF1–3′A, 4 with SDF1–wild-type). High level SDF-1 expression was observed in 15 of 34 children with SDF1–3′A as compared with 16 of 50 with wild type (P < 0.03). More notably, the children with AIDS-NHL had significantly elevated levels of SDF-1 mRNA in peripheral blood mononuclear cells, obtained at the time of presentation in 10 children and 8.5 to 19.4 months before (median, 15 months) in 7 children, as compared with the children in the non-NHL group (P < 0.00001). The amounts of cell-associated HIV-1 DNA and singly spliced HIV-1 mRNA were significantly greater in children with AIDS-NHL than those with non-NHL AIDS (P = 0.0052 and 0.011, respectively; stratified by antiretroviral treatment regimen), whereas their serum HIV RNA levels were comparable. Overexpression of SDF-1 and aberrant HIV-1 expression in circulating lymphocytes appear to be linked to the development of AIDS-lymphoma. Additional studies are required to determine whether excessive SDF-1, together with virally encoded factors, is directly involved in the pathogenesis of AIDS-lymphoma.

INTRODUCTION

Individuals with congenital or acquired immunodeficiency, including HIV-1-induced AIDS, are at greatly increased risk of developing NHL (1-5). Although these lymphomas occurring in the immunodeficient hosts have many common features (6), there are distinct differences between HIV-1-associated versus unassociated NHLs, e.g., approximately 20-40% of AIDS-NHL in adults are Burkitt or Burkitt-like lymphoma (7-9), in contrast to NHL seen in HIV-seronegative immunosuppressed hosts, who rarely develop Burkitt type lymphoma (10). In the pediatric population, Burkitt and diffuse large B-cell lymphomas are equally prevalent in children with AIDS (11), whereas Burkitt and lymphoblastic lymphoma of T-cell origin predominate in the general population (12). Moreover, with the exception of body-cavity-based lymphoma and primary central nervous system lymphoma that are uniquely linked to Kaposi sarcoma-associated herpes virus (13) and EBV (14, 15), respectively, the role of virally induced transforming events in the lymphomagenesis is not as firmly established in the majority of AIDS-NHL, as compared with NHL occurring in individuals with organ transplants, Wiskott-Aldrich syndrome, or X-linked lymphoproliferative syndrome, virtually all of which are driven by EBV (10). These dissimilarities between AIDS-associated versus non-AIDS-associated NHLs may suggest that certain HIV-1-induced pathophysiological elements are involved in the pathogenesis of AIDS-NHL in the setting of underlying immunodeficiency.

SDF-1, a principal ligand for HIV-1 coreceptor CXCR4 and CXCR7, receptor 4 (16, 17), was originally isolated from murine bone marrow stromal cell line as pre-B-cell growth-promoting factor (18) and is believed to play a critical role in the B-cell development (18-21). SDF-1 consists of two subtypes, SDF-1α and SDF-1β, which arise from a single gene by an alternative splicing and share identical amino acid sequences except for four additional amino acid residues that are present in SDF-1β (22). A common polymorphism in the 3′ untranslated region of the SDF-1β gene transcript, abbreviated as SDF1–3′A, has been associated with delayed onset of AIDS in HIV-1-infected SDF1–3′A/A homozygotes (23), although others (24, 25) have reported its association with HIV disease acceleration. The genetic influences of SDF1–3′A on AIDS or AIDS-related illnesses are complex and probably confounded by other genetic elements (26-29).

We have shown recently (30) that both homozygous and heterozygous SDF1–3′A variants were associated with an increased risk of NHL in HIV-1-infected individuals, whereas a heterozygous 32-bp deletion (Δ32) of CC-chemokine receptor 5 (CCR5) encoding gene, another major coreceptor for HIV-1 (31, 32), decreased its risk. This striking association between SDF1–3′A genotype and the risk of NHL was not observed in uninfected individuals (30). It has been demonstrated (33-40) that CCR5 Δ32 heterozygotes progress to AIDS at a much slower rate and harbor a significantly lower viral load because of defective expression of CCR5. Therefore, the protective effect of CCR5 Δ32 genotype against AIDS-NHL may be mediated through better preserved immune surveillance and/or reduced chronic immune stimulation by HIV-1 or other pathogens. However, the mechanisms by which the SDF1–3′A polymorphism influences AIDS-related lymphomagenesis are not as clear. The simplest hypothesis is that this gene variant increases the level of SDF-1 mRNA, because the polymorphism is located within the 3′ untranslated region of the transcript that is involved in the regulation of mRNA turnover (41-44). Thus, the effect of SDF1–3′A on AIDS-lymphomagenesis may be brought forth via enhanced expression of SDF-1, especially in the setting of HIV-1 infection. In the current study, we investigated the levels of SDF-1 mRNA in PBMCs obtained from HIV-1-infected children in various stages of HIV-1 disease and asked whether the genetic linkage of SDF1–3′A with AIDS-lymphomagenesis involves an altered expression of the chemokine.
MATERIALS AND METHODS

Patients and Clinical Specimens. Eighty-four HIV-1-infected children ranging in age from 0.7 to 18 years (median, 5.8) were studied. These children had been enrolled in various clinical protocols at the HIV and AIDS Malignancy Branch, NCI, between April 1987 and January 1997 and had previously cryopreserved PBMCs and sera available for the study. Informed consent was obtained from each study subject or his/her guardian for a participation in the NCI clinical protocols, which were approved by the Institutional Review Board.

The study cohort included 12 children who developed NHL during the course of HIV-1 infection (AIDS-NHL group). Characteristics of these children are summarized in Table 1 and elsewhere (45). Table 1 also lists an additional child with AIDS-NHL (patient 13), who had fresh lymphoma tissue available for the study (see below). PBMCs collected at the time of clinical diagnosis of NHL (−1.8 to 9.6 months; median, 0.3; in relation to the date of diagnosis) were available for the RNA evaluations in 10 patients (patients 1, 2, 4, 5, 6, and 8–12). PBMC specimens collected 8.5 to 19.4 months (median, 15) before the diagnosis of NHL (pre-NHL) were available in patients 1, 3, 6, 7, 8, 10, and 12. PBMC specimens for DNA evaluations were available in all of the patients (−20.6 to 9.6 months; median, 0.2; in relation to the NHL diagnosis), although the quantity was not sufficient to carry out HIV-1 DNA quantitation (see below) in patient 1. Serum samples from the time of diagnosis (−1.6 to 1.4 months; median, −0.2) were available in all of the patients but patient 9. Additional serum samples collected 11.2 to 17.2 months (median, 12.6) before the NHL diagnosis were available in nine patients (patients 1−6, 8, 10, and 12). In patients 1, 3, 6, 8, 10, and 12, who had both PBMC and serum samples available for the pre-NHL evaluation, differences in the timing of specimen collections were within ±3 months for each individual, except in patient 12 whose pre-NHL PBMC and serum samples had been obtained 19.4 and 11.6 months before the NHL diagnosis. Fresh lymphoma tissues from two children with AIDS-NHL (patients 4 and 13) were available for the RNA evaluation.

Of the remaining 72 children (non-NHL group), 41 had AIDS-defining conditions, other than lymphoma, according to the Centers for Disease Control and Prevention pediatric HIV classification system (46) at the time study specimens were collected (non-NHL AIDS group), whereas 31 children were still asymptomatic or moderately symptomatic (pre-AIDS group). Among the 41 children in the non-NHL AIDS group, six had a history of biopsy-proven conditions, other than lymphoma, according to the Centers for Disease Control and Prevention pediatric HIV classification system (46) at the time study specimens were collected (non-NHL AIDS group), whereas 31 children were still asymptomatic or moderately symptomatic (pre-AIDS group). Among the 41 children in the non-NHL AIDS group, six had a history of biopsy-proven polymorphic lymphoproliferative disorders (6). All of the 72 children were followed until death or for at least 2 years after the sample collection (median, 4 years) and confirmed to be lymphoma-free during the follow-up period. The follow-up cutoff date was March 31, 1999.

The majority of children had been on continuous, steady antiretroviral treatment regimens before the study specimens were collected between June 1987 and August 1997. Although most of the regimens consisted of nucleoside reverse transcriptase inhibitors either as a single agent or in combination, three children received protease inhibitor as a single agent while they were enrolled in the NCI clinical protocols (47, 48). However, this protease inhibitor-based antiretroviral regimen was not currently recommended highly active antiretroviral therapy because of the investigational nature of the Phase I/I protocols (47, 48).

PBMCs were also obtained from 66 normal blood bank donors. Randomly selected donor PBMCs, which contained high-level SDF-1 mRNA, were subjected to a separation of T (CD3+), B (CD19+) cells, and monocytes (CD14+) by magnetic beads, using methods described previously (49) that yielded 95–99% purity of each cell population. Fresh lymph nodes or tonsil tissues, obtained from three HIV-1-infected children in the non-NHL group when diagnostic or therapeutic interventions were required, were available for the RNA evaluation.

Nucleic Acid Extraction and Reverse Transcription of RNA. Genomic DNA was extracted from PBMCs as described (50). Total cellular RNA was extracted from PBMCs, lymphoid tissues, or Burkitt lymphoma cell lines A5283, AG876, PA682PB, P3HR-1, BJAB, Akata, Raji, Namalwa, and Louckes (kind gifts from Dr. Kishor Bhatia, NCI) using Total RNA Isolation kit (5 Prime-3 Prime, Inc., Boulder, CO) following by treatment with RNase-free DNase I (Promega, Madison, WI). The concentrations of DNA and RNA were determined by absorbance readings at a wavelength of 260 nm, measured by spectrophotometer.

Two μg of DNase-treated sample RNA were subjected to cDNA synthesis as described previously (50, 51) in a final volume of 20 μl of reaction mixture, containing 3 mM MgCl2, 75 mM KCl, 50 mM Tris-HCl, 50 μM deoxyribonucleotide triphosphates (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), 10 mM DTT (Life Technologies, Inc., Rockville, MD), 20 units of RNasin (Promega), 500 ng of random primers (Promega), and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The mixture was incubated at 42°C for 45 min, followed by 2-min incubation at 95°C, and synthesized cDNA was used for subsequent gene amplifications. Equivalent amounts of DNase-treated, non-reverse-transcribed RNA samples were included in all of the PCR experiments to ensure that each cDNA sample was free of genomic DNA contamination.

Genotype Analysis. A portion of each PBMC DNA was used to determine the SDF-1 genotype by PCR combined with an MspI-RFLP as described previously (23). Both heterozygous (3′A/3′A) and homozygous (3′3′A) SDF-1 gene variants are referred to as SDF1–3′A in the current article unless otherwise specified.

Table 1  Characteristics of 13 HIV-1-infected children with NHL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Race</th>
<th>SDF-1 genotype</th>
<th>Age at NHL (yr)</th>
<th>Transmitted (yr)</th>
<th>Sites of disease</th>
<th>Histology</th>
<th>Phenotype</th>
<th>EBV in tumor</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>W</td>
<td>3′/A/wt</td>
<td>13.3</td>
<td>13.3</td>
<td>Small bowel</td>
<td>LCL</td>
<td>B cell</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>B</td>
<td>3′/A/mt</td>
<td>5.5</td>
<td>5.5</td>
<td>Multiple bones and sinus</td>
<td>Burkitt type</td>
<td>B cell</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>W</td>
<td>wt/wt</td>
<td>3.3</td>
<td>3.3</td>
<td>Peripheral, mediastinal and abdominal LN</td>
<td>Plasmacytoid</td>
<td>B cell</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>W</td>
<td>3′/A/mt</td>
<td>15.2</td>
<td>10.3</td>
<td>Lung</td>
<td>MALT type</td>
<td>B cell</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>W</td>
<td>wt/wt</td>
<td>1/13.7</td>
<td>7.3</td>
<td>Nasopharynx</td>
<td>Burkitt type</td>
<td>B cell</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>W</td>
<td>wt/wt</td>
<td>18.3</td>
<td>10.7</td>
<td>Gastric mucosa</td>
<td>MALT type</td>
<td>B cell</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>W</td>
<td>3′/A/mt</td>
<td>1.7</td>
<td>1.7</td>
<td>CNS</td>
<td>LCL</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>W</td>
<td>3′/A/mt</td>
<td>12.2</td>
<td>9.7</td>
<td>Cervical LN</td>
<td>Burkitt type</td>
<td>B cell</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>B</td>
<td>wt/wt</td>
<td>1.0</td>
<td>1.0</td>
<td>Skin, peripheral LN, Mediastinum, pericardium, spleen, lung and mandible</td>
<td>LCL/N</td>
<td>T cell</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>B</td>
<td>3′/A/mt</td>
<td>7.1</td>
<td>7.1</td>
<td>Lung</td>
<td>MALT type</td>
<td>B cell</td>
<td>Positive</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>W</td>
<td>3′/A/mt</td>
<td>12.7</td>
<td>12.7</td>
<td>Mandible</td>
<td>LCL/N</td>
<td>non-B, non-T</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>W</td>
<td>3′/A/mt</td>
<td>11.2</td>
<td>11.2</td>
<td>Orbit, sinus, long bones, and abdomen</td>
<td>Burkitt type</td>
<td>B cell</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>B</td>
<td>wt/wt</td>
<td>4.3</td>
<td>4.3</td>
<td>Inguinal LN</td>
<td>LCL/IBL</td>
<td>B cell</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* The children had acquired HIV-1 infection perinatally, except for patients 4, 5, 6, and 8, who were infected via transfusion of blood components.

a M, male; F, female; W, white; B, black; Dx, diagnosis; LN, lymph node; CNS, central nervous system; LCL, large cell lymphoma; MALT type, malignant lymphoma of mucosa associated lymphoid tissue type; AN, anaplastic; IBL, immunoblastic; N/A, information not available.

b By in situ hybridization.

c This patient developed two histologically and immunophenotypically distinct NHLs, confirmed by molecular genetic studies of immunoglobulin gene rearrangement.

INCREASED SDF-1 mRNA LEVELS IN PBMCs OF CHILDREN WITH AIDS-NHL

5029
Evaluation of SDF-1 mRNA by PCR. The levels of SDF-1α and SDF-1β mRNA were evaluated by RT-PCR, using two primer pairs to distinguish each transcript: α-forward (5'-TAGCCCGCTGAAGAACACAA-3') and α-reverse (5'-CCAGGGGACAGGAGGATG-G'-3'), generating a 511-bp fragment from the third exon; β-forward (5'-CATGCACTGCGGCAGAAGGC-3') and β-reverse (5'-AGCTTGTGCTCAGGATCC-3'), generating a 302-bp fragment from the fourth exon (22, 23). SDF-1 mRNA refers to SDF-1α and SDF-1β mRNA unless otherwise specified throughout the article. PCR was performed with cDNA derived from 0.2 μg of RNA for 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s followed by a final extension at 72°C for 7 min. For the semiquantitative assessment of SDF-1β mRNA levels (see below), the cycling number was reduced to 30 because the results from preliminary experiments demonstrated that the PCR with 35 cycles reached the plateau effect for samples with high-level SDF-1β mRNA. The amplified products were subjected to DNA sequencing to verify the source of respective transcripts. Each experiment consisted of 30-cycle PCR using a G3PDH 5’ upstream and 3’ downstream primer pair (Clontech Laboratories, Palo Alto, CA) to ensure that the amounts of input cDNA were optimum among tested samples. The samples that did not give rise to clear G3PDH PCR products were omitted from the analyses. Conditions for all of the PCR assays were determined to be suitable for linear phase amplification. The PCR products were visualized by electrophoresis on 2% agarose gel, and the signal intensity of each fragment was measured by Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The levels of SDF-1α and SDF-1β mRNA were normalized by the signal intensity of G3PDH PCR products and expressed by the intensity ratio.

Analysis of HIV-1 DNA and RNA. The amounts of HIV-1 DNA in PBMCs were evaluated by quantitative PCR using a primer pair SK38 and SK39 as described previously (50, 52) and corrected by CD4% to estimate the normalized by the signal intensity of G3PDH PCR products and expressed by the intensity ratio. The PCR products were visualized by electrophoresis on 2% agarose gel, and the signal intensity of each fragment was measured by Kodak Digital Science Dynamics, Sunnyvale, CA). The levels of SDF-1α and SDF-1β mRNA were normalized by the signal intensity of G3PDH PCR products and expressed by the intensity ratio.

Detection of EBV DNA in PBMCs. The presence of EBV DNA in PBMCs was evaluated by nested PCR as described previously (60). Genomic DNA obtained from AA-2 cells, EBV+ B lymphoblastoid cell line (Ref. 61; AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH; contributed by Dr. Michael Hershfield), was used as a positive control for each experiment. This nested PCR detected at least one AA-2 cell mixed in 10^6 PBMCs of an EBV-negative donor. Every sample was tested at least twice in different experiments, and the sample was considered positive for EBV if a positive result could be obtained at least once.

Statistical Analysis. The exact Kruskal-Wallis test and Wilcoxon’s rank-sum test (two-sided) were used for nonparametric analyses to compare the levels of SDF-1 mRNA, CD4%, serum HIV-1 RNA, cell-associated HIV-1 DNA, and HIV-1 mRNA among various groups defined by genotype or disease status. When appropriate, the stratification by various antiretroviral regimens was applied to the Wilcoxon’s rank-sum test for two-group comparisons. Spearman’s rank correlation coefficient was computed to assess the relationship between levels of SDF-1α versus SDF-1β mRNA or between SDF-1α mRNA versus cell-associated HIV-1 mRNA levels. Fisher’s exact test was used to compare the proportion of individuals with high-level or detectable SDF-1 mRNA in PBMCs between SDF1-wild-type versus S3’A carriers or between the normal donors and HIV-1-infected children, as well as to assess the relationship between the prevalence of EBV DNA in PBMCs and various HIV-1-associated disease conditions among the study subjects.

RESULTS

Levels of SDF-1 mRNA in Normal Donor PBMCs and Various Lymphoid Tissues. We first investigated whether SDF-1 mRNA could be detected in PBMCs of the normal donors. SDF-1α and SDF-1β mRNA were distinctly demonstrated in PBMCs from 19 of 66 (29%) individuals examined (bright PCR bands; Fig. 1A). Of the remaining 47 donors, samples from 19 donors contained borderline low levels of SDF-1α and/or SDF-1β mRNA observed as dim PCR products, whereas 28 individuals had no detectable SDF-1α mRNA in PBMCs. Bright SDF-1 RT-PCR product bands were observed in 9 of 33 individuals with SDF1-wild-type and 10 of 33 with SDF1–3’A polymorphism (8 heterozygotes and 2 homozygotes; not significant), demonstrating no clear association between high SDF-1 expression and SDF1–3’A genotype in the normal donors. Levels of SDF-1 mRNA were compared in fractionated cell populations from randomly selected PBMC samples that yielded abundant SDF-1 RT-PCR products. SDF-1 mRNA was invariably detected in T lymphocytes and monocytes (Fig. 1A). B lymphocytes also expressed SDF-1 in some donors, but to a lesser extent than T-cell and monocyte populations. In additional experiments, a pair of PBMC samples collected between 4 to 11 months apart from each of three normal donors were subjected to the determination of SDF-1 mRNA levels. Although one donor (SDF1-wild-type) consistently showed negative SDF-1 expression, two other individuals (SDF1-wild-type and SDF1–3’Awt) had negative results with one sample and positive (clearly and faintly visible) results with the other, suggesting a certain degree of fluctuation in the SDF-1 mRNA levels in some donors.

The amounts of SDF-1 mRNA were next examined in various lymphoid tissues obtained from HIV-1-infected children (Fig. 1B). Tonsil tissues from three uninfected children with tonsil hypertrophy served as a reference. Regardless of HIV-1 infection status or the presence of malignant cells, all of the lymphoid tissues examined contained large amounts of SDF-1 mRNA. The majority of Burkitt lymphoma cell lines (eight of nine tested), including those derived from two cases of AIDS-related lymphoma (AS283 and PA682PB), did not express SDF-1 (Fig. 1B).

SDF-1 mRNA Levels in PBMCs from HIV-1-infected Children. The allele frequency of SDF1–3’A in the current cohort of HIV-1-infected children was consistent with data reported previously (23) from HIV-1-infected adult cohorts. Detailed analyses on the relationship between SDF1–3’A genotype and overall disease progression patterns for the pediatric cohort are reported elsewhere (29). At the time PBMCs for SDF-1 mRNA evaluations were obtained, children in the NHL group tended to have lower CD4% than those with non-NHL AIDS, although it was not statistically significant (mean ± SD, 10.0 ± 10.8 versus 15.1 ± 13.6 for NHL versus non-NHL AIDS groups, respectively; P = 0.28). Eight of 12 children with AIDS-NHL carried SDF1–3’A polymorphism (Table 1). In 25 of 84 HIV-1-infected children (30%), both SDF-1α and SDF-1β were abundantly expressed in PBMCs (bright PCR bands), whereas an additional 42 children (50%) had low levels of SDF-1α and/or SDF-1β mRNA in the PBMCs (dim PCR bands). The proportion of the subjects with detectable SDF-1 mRNA in PBMCs (bright or dim PCR bands)
INCREASED SDF-1 mRNA LEVELS IN PBMCs OF CHILDREN WITH AIDS-NHL

Fig. 1. Gel electrophoresis of SDF-1α, SDF-1β, and G3PDH PCR products in the normal donor PBMCs, lymphoid tissues, and Burkitt lymphoma cell lines. A, shown are representative profiles of SDF-1 mRNA expression in the normal donor PBMCs grouped by SDF-1 genotype: homozygous wild type (SDF1-wt/wt), heterozygous variant (SDF1-3′A/wt), and homozygous variant (SDF1-3′A/3′A). PBMCs from two to three donors with positive SDF-1 expression in each genotype were further subjected to lymphocyte subset separation. T lymphocytes (T) and monocytes (M) appeared to contain invariably higher levels of SDF-1 mRNA than B lymphocytes (B). B, SDF-1 mRNA were abundantly present in lymphoid tissues obtained from HIV-negative children as well as from HIV-infected children. Also included are tissues from two children with AIDS-related lymphoma (patients 4 and 13; Table 1). In contrast to the lymphoid tissues, SDF-1 mRNAs were rarely expressed in Burkitt lymphoma cell lines including those from AIDS-NHL (AS283 and PA682PB). Arrows, pertinent PCR products.

appeared to be higher in HIV-infected children (total, 67 of 84; 80%) than in the normal donor population (total, 38 of 66; 58%; P = 0.004). Moreover, in contrast to the normal donor group, high SDF-1 expression was observed in 15 of 34 HIV-infected children with SDF1-3′A as compared with 10 of 50 children with SDF1-wildtype (P < 0.03).

To further examine the SDF-1 expression profile in the HIV-infected children, the band intensities of SDF-1α and SDF-1β RT-PCR products normalized by G3PDH PCR were compared among the study subjects. Overall, there was a strong correlation between the signal intensities of SDF-1α and SDF-1β products (r = 0.71; P < 0.0001). The levels of SDF-1α and SDF-1β expression did not significantly differ between the pre-AIDS and non-NHL AIDS groups (Fig. 2A). Strikingly, 10 of 10 (100%) children with AIDS-NHL had significantly higher levels of SDF-1α and SDF-1β mRNA in PBMCs obtained at the time of NHL diagnosis, as compared to those without lymphoma (non-NHL group; P < 0.00001 for both SDF-1α and β mRNA; Fig. 2B and 3). PBMCs from patients 1, 3, 6, 7, 8, 10, and 12 collected at 8.5 to 19.4 months (median, 15) before the diagnosis of NHL (pre-NHL) had equally high levels of SDF-1 mRNA (Fig. 2B and 3), suggesting that the increased expression of SDF-1 in PBMCs preceded clinically overt manifestation of lymphoma by at least 1 to 2 years in these children. In four children in the pre-AIDS (n = 2) and AIDS (n = 2) groups, who were tested positive for SDF-1 expression in PBMCs, additional PBMC specimens obtained at 4.9 to 17 months after the original test specimens also showed comparable levels of SDF-1α and β expression (data not shown). There were no significant differences in the levels of SDF-1α and β mRNA by the administration of antiretroviral treatment (68 treated versus 16 untreated) or by the treatment regimens at the time of specimen collection (47 monotherapy versus 21 combination therapy; data not shown). In general, there were no discernible differences in the PBMC SDF-1 mRNA levels among the children with lymphomas of different histopathology or with EBV-positive versus EBV-negative lymphomas (Table 1). Of note, patient 9, who developed a distinct T-cell lineage NHL, had the lowest levels of SDF-1α and SDF-1β mRNA within the AIDS-NHL group (Fig. 3).

Presence of EBV DNA in PBMCs in Children with or without AIDS-NHL. EBV DNA was detected in PBMCs from 49 of 84 (58.3%) study subjects overall; 21 of 33 children in the pre-AIDS group, 21 of 39 with non-NHL AIDS, and 7 of 12 with AIDS-NHL (not significant; Table 2). It should be noted that 2 of 41 children classified in the non-NHL AIDS group for the mRNA evaluation were included in the pre-AIDS group for the EBV evaluation, because they were still asymptomatic when the PBMC samples for EBV DNA testing were collected. All of the six children with lymphoproliferative disorders (subgroup of non-NHL AIDS) tested positive for EBV DNA in PBMCs. The presence of EBV DNA in PBMCs was not associated with the development of NHL (Table 2) or higher levels of SDF-1 expression (data not shown).

HIV-1 Viral Load in Children with versus without AIDS-NHL. Because many of the children had been on steady but various antiretroviral treatment regimens before the study specimen collections as discussed earlier, the levels of HIV-1 viral load were compared with the stratification by the administration of various antiretroviral treatment regimens (12 untreated, 51 monotherapy, and 19 combination therapy for serum HIV-1 RNA evaluation; 26 untreated, 37 monotherapy, and 19 combination therapy for HIV-1 DNA evaluation). The children in the pre-AIDS group had significantly lower levels of serum viremia (mean ± SD, 3.73 ± 0.85 log10 copies/ml) and CD4+ T-cell-associated HIV-1 DNA (3.17 ± 0.66 log10 copies/10⁶ CD4+ T cells) as compared with those who had non-NHL AIDS (4.73 ± 0.91 and 3.85 ± 0.87 for serum HIV-1 RNA levels and CD4+ T-cell-associated HIV-1 DNA, respectively; P = 0.00004 and 0.00008, stratified by antiretroviral therapy, respectively; Fig. 4, A and B). Notably, the children in the NHL group had significantly greater
amounts of CD4+ T-cell-associated HIV-1 DNA (4.51 ± 0.48) than those with non-NHL AIDS (P = 0.0052; stratified by antiretroviral therapy; Fig. 4B), whereas their serum HIV-1 RNA levels were comparable (4.45 ± 1.10; Fig. 4A). HIV-1 DNA evaluation for patients 2, 4, and 7 were performed using pre-NHL PBMC samples obtained 7 to 20 months before the NHL diagnosis. The exclusion of these three patients did not alter the analysis result (data not shown). Additional serum samples collected 11.2 to 17.2 months (median, 12.6) before the NHL diagnosis in nine patients (patients 1–6, 8, 10, and 12) demonstrated similar levels of serum HIV-1 RNA to those at NHL diagnosis (data not shown).

HIV-1 generates three size classes of HIV-1 RNAs, small (multiply spliced), intermediate (singly spliced), and full length (unspliced), from the template provirus integrated in the host genome (62). Although higher numbers of proviral copies can theoretically produce larger amounts of HIV-1 RNA, the ratio of synthesized unspliced versus spliced HIV-1 mRNA may not remain constant through various stages of HIV disease (63–66). Furthermore, the amounts of unspliced HIV-1 RNA detected in plasma or serum only reflect the rate of de novo virion production (67), not necessarily the extent or dynamic profile of viral gene expression. The differential levels of cell-associated proviral burden, despite comparable levels of serum HIV-1 RNA load, noted between non-NHL AIDS versus AIDS-NHL groups prompted us to examine the relative abundance of unspliced and spliced PBMC-associated HIV-1 mRNA for the study subjects who had additional RNA samples available for the assays (n = 60).

The children in the AIDS-NHL group (n = 9) had relatively high levels of HIV-1 mRNA in PBMCs obtained at the time of the NHL diagnosis (patients 2, 5, 6, 9, and 12) or 8.5 to 19.4 months before (patients 1, 3, 6, 7, 8, and 12), in particular HIV-1 mRNA species that could be detected by the T1-T2 primer pair (Fig. 5A). The signal intensities of T1-T2 PCR products were significantly higher in the AIDS-NHL group than in the non-NHL AIDS group (n = 26; P = 0.011; stratified by antiretroviral therapy), whereas the levels of SK38-SK39 PCR products were not considerably different between the two groups (P = 0.15; stratified by antiretroviral therapy; Fig. 5, B and C). Moreover, the levels of T1-T2-amplified products appeared...
to correlate with the amounts of SDF-1 mRNA ($p = 0.44$; $P = 0.0006$), because of a distinct cluster of the data representing the children with B-cell lineage NHL, who had significantly elevated levels of both SDF-1 and HIV-1 expression in PBMCs (Fig. 6).

**DISCUSSION**

NHL is the most or second most common cancer occurring in children or adults with HIV-1-infection (68). Heterogeneous molecular characteristics of AIDS-NHL described by a number of previous studies (69–71) suggest that accumulation of various transforming events is probably responsible for the ultimate formation of tumor progenitor cells at different stages of B-cell maturation, most dominantly at germinal center or postgerminal center stages (72). However, whether there are common etiological determinants that provoke B-cell malignant transformation in HIV-1-infected individuals, other than acquired immunodeficiency, has yet to be determined. In recent years, widespread use of highly active antiretroviral therapy has been credited for a substantial reduction in the incidence of AIDS-related death or opportunistic illnesses, including Kaposi sarcoma (73–78). Yet, its impact on the occurrence of AIDS-NHL has only been modest to nonsignificant (75–79). The reasons for a slower decline in the incidence of AIDS-NHL in the post-highly active antiretroviral therapy era are not clear, but improved immune surveillance and the reduction of chronic B-cell stimulation and proliferation that can result from well-controlled viral replication (80) are evidently insufficient to block the process of NHL tumorigenesis in HIV-1-infected individuals.

In the current study, we demonstrated that the children with AIDS-NHL, especially of B-cell lineage lymphomas, had significantly higher levels of SDF-1 mRNA in PBMCs, as compared with those who did not develop NHL for at least 2 years (median, 4 years) after the evaluation. The increased SDF-1 expression preceded clinical diagnosis of NHL by 1 to 2 years, suggesting its possible linkage to the early stages of lymphomagenesis. Our study was the first to demonstrate a biological condition invariably observed in individuals with B-cell lineage AIDS-NHL. The SDF-1 expression in PBMCs has been reported previously (22, 81) to be negligible in normal population. However, we found that SDF-1 mRNA may sporadically be detected in some normal donor PBMCs, commonly in T lymphocytes and monocytes. High-level expression of SDF-1 in peripheral blood-derived cells has been demonstrated in T cells obtained from carriers of human T-cell leukemia virus type 1 (82) or phytohemagglutinin-stimulated PBMCs of normal donors (81). These findings suggest that SDF-1 may be up-regulated via activation or inflammatory stimuli, especially in T lymphocytes. Therefore, certain infectious diseases, such as HIV-1 infection, could be associated with increased expression of SDF-1 in PBMCs, as seemingly corroborated by our observation that a greater proportion of HIV-1-infected children had detectable SDF-1 mRNA in PBMCs compared with that of the normal volunteer population.

Although our data do not directly address whether overexpression of SDF-1 triggers lymphomagenesis in HIV-1-infected individuals, the findings have an intriguing implication that arises from the biological functions of SDF-1 recognized to date. The development of B cells is initiated in the bone marrow from B-cell progenitors by a process of distinct gene rearrangements called V(D)J recombination (83–85). The majority of pre-B cells remain in the bone marrow while completing a series of gene rearrangements and editing, eventually developing to mature naïve B cells, which will then migrate to the germinal centers of secondary lymphoid organs. SDF-1 secreted from bone marrow stromal cells is believed to regulate pre-B-cell trafficking within the optimal bone marrow microenvironment as a chemoattractant, as well as to promote proliferation and differentiation of B-cell progenitors as a growth factor. It is conceivable that aberrant expression of SDF-1 outside of bone marrow, such as in the lymphoid organs or the areas of lymphocyte infiltration in various nonlymphoid organs commonly found in HIV-1-infected individuals (50), may induce enhanced emigration of pre-B cells into a suboptimal environment, possibly predisposing the cells to dysregulated proliferation and gene rearrangements. After migrating to germinal centers of secondary lymphoid tissues, B cells usually undergo three types of gene

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**Table 2: Detection of EBV DNA in PBMCs obtained from HIV-1-infected children at various disease stages**

<table>
<thead>
<tr>
<th>PBMC EBV PCR</th>
<th>pre-AIDS</th>
<th>non-NHL</th>
<th>AIDS</th>
<th>LPD*</th>
<th>NHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV DNA +</td>
<td>21</td>
<td>15</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>EBV DNA –</td>
<td>12</td>
<td>18</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total no. (% positive)</td>
<td>33 (63.6)</td>
<td>33 (45.5)</td>
<td>6 (100)</td>
<td>12 (58.3)</td>
<td></td>
</tr>
</tbody>
</table>

* LPD, lymphoproliferative disorders (see text); +, positive; –, negative.

* No NHL group (pre-AIDS + non-NHL AIDS + LPD) vs. NHL group: not significant.

* Not significant.

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![Graph A](image1.png)

**Graph A:** Comparison of serum HIV-1 RNA (A) and CD4+ T-cell-associated HIV-1 DNA levels (B). Children in the pre-AIDS group had significantly lower levels of serum HIV-1 RNA and CD4+ T-cell-associated HIV-1 DNA than those who had AIDS ($p = 0.00004$ and 0.00008, respectively; stratified by antiretroviral therapy). Children with AIDS-NHL appeared to have higher levels CD4+ T-cell-associated HIV-1 DNA than non-NHL AIDS patients ($p = 0.0052$; stratified by antiretroviral therapy), whereas their serum HIV-1 RNA levels were comparable. In 3 of 11 children with AIDS-NHL who had HIV-1 DNA levels evaluated, the specimens were available only from the time of pre-NHL (see text) and are shown with asterisks (*). Of all the serum samples for the AIDS-NHL group shown are from the time of diagnosis. Also available but not included were serum samples obtained before the diagnosis (−17.2 to −11.2 months; median, −12.6) in nine children, which showed similar levels of serum HIV-1 RNA to those at NHL diagnosis. Bars, geometric means.
INCREASED SDF-1 mRNA LEVELS IN PBMCs OF CHILDREN WITH AIDS-NHL

Fig. 5. Comparison of HIV-1 mRNA expression patterns in PBMCs obtained from children in the non-NHL versus NHL groups. A, shown are representative gel electrophoresis results from both groups. RNA samples extracted from normal donor PBMCs infected with two different clinical isolates (not from the current study subjects) were included in each experiment as a reference (Control A and Control B). Arrows, respective PCR products. PBMC samples obtained before the clinical diagnosis of NHL (pre-NHL) were available from patients 6 and 12 and are shown in the left lane for each patient as a comparison. +, PBMC samples for HIV-1 mRNA analysis were only available from pre-NHL for patients 1, 3, 7, and 8 (see text). B and C, band intensities of SK38-SK39 PCR (B) and T1-T2 PCR products (C) were adjusted by G3PDH-PCR band densities and compared by the disease status. Similar to the findings from serum HIV-1 RNA and CD4+ T-cell-associated HIV-1 DNA comparisons, children in the pre-AIDS group had significantly lower levels of HIV-1 mRNA in PBMCs. Children with AIDS-NHL had significantly higher levels of T1-T2 amplified products ($P = 0.011$; stratified by antiretroviral therapy), whereas their unspliced HIV-1 mRNA quantities (SK38-SK39 PCR products) were comparable with those in the non-NHL AIDS group. The pre-NHL samples are shown with + as in A. Bars, median values.

Fig. 6. Relationship between SDF-1 mRNA levels and HIV-1 mRNA amplified by the T1-T2 primer pair. The data points for the children in NHL group cluster in the right upper quadrant, indicating they had higher levels of both SDF-1 mRNA and HIV-1 mRNA in PBMCs. Arrow, the data from patient 9 with NHL of T-cell phenotype (Table 1), isolated from the rest of the children in the NHL group, +, as discussed in the Fig. 5 legend, PBMC samples for HIV-1 mRNA analysis were only available from pre-NHL for patients 1, 3, 7, and 8. Therefore, the levels of SDF-1 mRNA from corresponding pre-NHL PBMC specimens are used for the plot in these cases.

modifications: somatic hypermutations, class switching, and receptor editing. This process of DNA modifications by itself can present another risk for malignant transformation. Prematurely emigrated pre-B cells may be even more vulnerable to faulty gene modifications or other transforming events, further increasing the chance of B-cell tumorigenesis. Additionally, SDF-1 may be capable of enhancing the chance of survival of malignant B cells, as has been demonstrated previously (86–89) with B-lineage acute lymphoblastic leukemia, which shares the immunophenotypic and molecular characteristics with Burkitt lymphoma.

It is of note that the greater number of HIV-1-infected children with SDF1–3’A polymorphism had high SDF-1 expression in PBMCs than those with SDF1-wild-type. The data suggest that the presence of HIV-1 infection may induce up-regulation of SDF-1 mRNA in individuals with SDF1–3’A genotype and may corroborate our previous observation that HIV-1-infected adults and children with SDF1–3’A gene variant had increased risk of AIDS-NHL (30). However, it is still unclear how the SDF1–3’A mutation influences the SDF1 expression levels in circulating lymphocytes of HIV-1-infected individuals. The SDF1–3’A genotype does not appear to be sufficient by itself, for there was no significant difference in the expression levels between individuals with SDF1-wild-type versus SDF1–3’A in the normal population. It is conceivable that the SDF1–3’A may be in a strong linkage disequilibrium with yet unidentified gene variant(s), which may regulate the expression of both SDF1α and SDF1β in the presence of inflammatory signaling. Regardless of the genetic mechanisms, our mRNA-based data must be interpreted with caution, because it has yet to be determined whether the increased level of SDF1 mRNA corresponds to the increased SDF1 protein or whether overexpression of SDF1 in PBMCs represents increased levels of SDF1 in the peripheral circulation and/or in lymphoid tissues. In the current study, the AIDS-associated lymphoma tissues were found to contain equally abundant, but not necessarily greater, amounts of SDF1 mRNA compared with the lymph nodes from HIV-1-infected and uninfected children. The abundant expression of SDF1 has been demonstrated previously (22, 90) in NHL tissues obtained from HIV-1-seronegative individuals, most likely reflecting the expression in nonmalignant cells in background. Additional studies that compare the levels of SDF1 protein in circulation and in tissues or its expression patterns between B-cell lymphoma versus nonmalignant lymphoid tissues will help elucidate the roles of SDF1 chemokine in AIDS-lymphomagenesis.

Higher levels of cell-associated HIV-1 DNA and HIV-1 mRNA observed in the children with AIDS-NHL may provide another important insight into the pathogenesis of AIDS-lymphoma. HIV-1 is not believed to be directly involved in the malignant transformation of B cells, because the viral genome is exclusively confined to infiltrating T lymphocytes within AIDS-lymphoma tissues, and lymphoma cell lines derived from AIDS-NHL do not contain HIV-1 (91, 92). However, in the current study, we found that the children with
AIDS-NHL appeared to have different patterns of HIV-1 gene expression. The T1-T2 primer pair can detect unspliced HIV-1 mRNA as well as intermediate size HIV-1 mRNAs, which encode Env, Vpr, Vif, Vpu, and one-exon Tat (56–62), whereas the gag primer pair, SK38-SK39, detects only unspliced HIV-1 mRNA. The difference noted in T1-T2 PCR products but not in SK38-SK39 PCR products between the AIDS-NHL versus non-NHL AIDS groups may reflect differential levels of singly spliced HIV-1 mRNA species between these two groups, implicating a potential involvement of virally encoded proteins in the pathogenesis of AIDS-NHL. Among the HIV-1-encoded proteins, Tat protein, a potent transactivator for viral gene expression, has been shown to be shed from infected T cells and taken up by surrounding bystander cells (93–96). Others have also reported that exogenous (extracellular) Tat protein can activate B cells (97) or enhance the germinal center B-cell proliferation (98). Because transcription of HIV-1 genome may persist despite complete suppression of plasma viremia by potent combination antiretroviral therapy (66), it is possible that certain types of virally encoded proteins, such as Tat, are continually produced within the lymphoid tissues of HIV-1-infected individuals, potentially influencing the regulatory process of B-cell differentiation, even after the initiation of potent antiretroviral treatment.

Emerging evidence (99–101) suggests that the impact of various chemokines on the HIV-1 replication cycle may range from viral suppression to enhancement, demonstrating the complex nature of chemokines on the HIV-1 replication cycle may range from viral suppression to enhancement, demonstrating the complex nature of chemokines on the HIV-1 replication cycle. It is tempting to speculate that a combination of excessive suppression to enhancement, demonstrating the complex nature of chemokines on the HIV-1 replication cycle may range from viral suppression to enhancement, demonstrating the complex nature of chemokines on the HIV-1 replication cycle.


Increased Level of Stromal Cell-Derived Factor-1 mRNA in Peripheral Blood Mononuclear Cells from Children with AIDS-related Lymphoma

Shizuko Sei, Dennis P. O'Neill, Sean K. Stewart, et al.


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