Inhibition of Purified CD34⁺ Hematopoietic Progenitor Cells by Human Immunodeficiency Virus 1 or gp120 Mediated by Endogenous Transforming Growth Factor β1

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Summary

Human CD34⁺ hematopoietic progenitor cells, stringently purified from the peripheral blood of 20 normal donors, showed an impaired survival and clonogenic capacity after exposure to either heat-inactivated human immunodeficiency virus (HIV) 1 (strain IIIB) or cross-linked envelope gp120. Cell cycle analysis, performed at different times in serum-free liquid culture, showed an accumulation in G0/G1 in HIV-1- or gp120-treated cells and a progressive increase of cells with subdiploid DNA content, characteristic of apoptosis. In blocking experiments with anti–transforming growth factor (TGF) β1 neutralizing serum or TGF-β1 oligonucleotides, we demonstrated that the HIV-1- or gp120-mediated suppression of CD34⁺ cell growth was almost entirely due to an upregulation of endogenous TGF-β1 produced by purified hematopoietic progenitors. Moreover, by using a sensitive assay on the CCL64 cell line, increased levels of bioactive TGF-β1 were recovered in the culture supernatant of HIV-1/ gp120–treated CD34⁺ cells. Anti-TGF-β1 neutralizing serum or TGF-β1 oligonucleotides were also effective in inducing a significant increase of the plating efficiency of CD34⁺ cells, purified from the peripheral blood of three HIV-1-seropositive individuals, suggesting that a similar mechanism may be also operative in vivo. The relevance of these findings to a better understanding of the pathogenesis of HIV-1–related cytopenias is discussed.

A common feature of the progression toward AIDS is that, besides the reduction in CD4⁺ T cell count, other peripheral blood cytopenias such as anemia, granulocytopenia, and thrombocytopenia invariably take place in up to 80% of HIV-1–seropositive subjects (1). The hematopoietic dysfunction in symptomatic HIV-1–seropositive subjects is underscored by an impaired in vitro growth capacity of either peripheral blood or bone marrow hematopoietic progenitor cells (2). Significantly, CD34⁺ cells purified from the bone marrow of AIDS patients also show poor colony-forming ability (3–6).

Although a variety of mechanisms have been claimed in the pathogenesis of peripheral blood cytopenias of AIDS patients (1), the role played by HIV-1 remains initially elusive. In fact, direct HIV-1 infection of CD34⁺ hematopoietic progenitor cells isolated from HIV-1–seropositive carriers has been reported only in a limited subset of cases and can hardly account for the functional impairment of hematopoiesis observed in these patients (3–9). Similarly, only a minority of purified CD34⁺ cells are susceptible to either productive or latent infection with HIV-1 in vitro (9–15). Therefore, the direct infection of hematopoietic stem/progenitor cells does not seem to be a leading cause for the observed pathophysiology, suggesting that mechanisms other than direct infection may be responsible for the AIDS-associated hematopoietic suppression.

In this context, we have previously shown that the in vitro exposure to either lymphocytotropic strains of HIV-1 (IIIB or ICR-3) or cross-linked gp120 significantly impaired the survival and growth of the TF-1 CD34⁺ hematopoietic cell line as well as bone marrow CD34⁺ cells (16). This suppressive effect appeared to be greatly dependent on the viral load, but took place in the absence of a productive or latent infection and was likely mediated by specific interactions of envelope gp120 with the CD4 antigen, expressed at low level on the surface of a subset of human hematopoietic progenitor cells (17, 18).

It was previously shown by single-cell cultures and limiting dilution analysis that early hematopoietic progenitor cells are able to produce autocrine TGF-β1 (19, 20), which

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is thought to play an essential role in the maintenance of the quiescence state of stem cells and more immature hematopoietic progenitors. Here we explore whether endogenous production of TGF-β1 could take part in the HIV-1 receptor expression. Here we explore whether anti-TGF-β1 serum could completely neutralize 10 ng of TGF-β1.

21mers corresponding to the antisense, sense, or missense sequences flanking the translation initiation regions of the mRNA for TGF-β1 were prepared as described by Hayter et al. (19). The sequence of the phosphorothioate oligonucleotides are as follows: TGF-β1 antisense, 5'-GGCGAGCGAGTGAGCGCGCGG-3'; TGF-β1 sense, 5'-CCCCCATGCGCGCTCCGGG-3'; TGF-β1 missense, 5'-GGGACCCGACTGACCGCGCGG-3'.

Isolation of CD34+ Progenitor Cells from Peripheral Blood. Informed consent for the study was obtained according to the Helsinki declaration of 1975 from 10 healthy donors and 3 HIV-1-seropositive subjects. Mononuclear cells were isolated from leukopheresis units (healthy donors) or 60 ml of peripheral blood (HIV-1-seropositive donors) by Ficoll-Paque (d = 1.077 g/ml; Pharmacia, Uppsala, Sweden), rinsed, and adherence-depleted overnight. Nonadherent cells were collected and aliquoted at a concentration of 25 x 10^6 cells/tube. 50 μl of the following mAbs were added to each tube: anti-CD2, anti-CD3, anti-CD8, anti-CD4, anti-CD11, anti-CD14, anti-CD19, anti-CD20 (Becton Dickinson & Co., San Jose, CA) in the presence of 0.5% BSA (fraction V, Sigma Chemical Co., St. Louis, MO). After two washings, 100 x 10^6 immunomagnetic beads, coated with anti-mouse IgG (MPC 450 Dynabeads; Dynal, Oslo, Norway) were then added to each tube to obtain an immunomagnetic bead/cell ratio of 10:1 in a final volume of 0.4 ml for 30 min in ice, under continuous agitation. Lineage-positive cells were removed by a magnet (MPC1 Dynabeads; Dynal) and the remaining cells were pelleted at a concentration of 5 x 10^6 cells/tube. After these negative selections, CD34+ cells were isolated using a magnetic cell sorting program (Mini-MACS; Miltenyi Biotec, Auburn, CA) and the CD34 isolation kit in accordance with the manufacturer's recommendations.

The purity of CD34-selected cells was determined for each isolation by flow cytometry using a mAb that recognizes a separate epitope of the CD34 molecule (HPCA-2; Becton Dickinson & Co.) followed by a goat anti-mouse IgG directly conjugated to fluorescein (GAM-FITC). CD34+ cells ranged ~95-98%. No differences in CD34 purity were observed in HIV-1-seronegative and -seropositive donors.

The presence of proviral DNA in CD34+ cells purified from HIV-1-seropositive subjects was examined by PCR, following a previously described procedure (7), with a sensitivity of 10 proviral copies in a background of 10^6 cells. Aliquots of 20,000 CD34+ cells were amplified with the HIV-1 gag-specific primers SK38-SK39. PCR runs included several reactions containing all reagents except DNA as negative controls, as well as HIV-1+ controls represented by H9 and Jurkat T cell lines chronically infected with HIV-1. At the end of the amplification reaction, 25-μl aliquots of the amplified products were resolved in a 3% agarose gel.

Materials and Methods

Growth Factors, Antibodies, and Oligodeoxynucleotides. rIL-3 and stem cell factor (sSCF), were purchased from Genzyme Corp. (Cambridge, MA). Erythropoietin (rEp) was kindly provided by Cilag (Milan, Italy). Purified TGF-β1 was purchased from R&D Systems, Inc. (Minneapolis, MN).

In neutralizing experiments, rabbit anti-TGF-β1 (R&D Systems, Inc.), rabbit anti-IFN-α (106 neutralizing units/ml; BioSource, Camarillo, CA) and rabbit anti-TNF-α (Genzyme Corp., Cambridge, MA) polyclonal sera were used. In preliminary experiments, 20 μl of anti-TGF-β1 serum could completely neutralize 100 ng of TGF-β1.

Virus Stock and Recombinant Viral Proteins. Virus stock was repre- sented by the supernatant of H9 lymphoblastoid T cells cultured at optimal cell density (0.5-1.5 x 10^6 cells/ml) and harvested 14 d after infection with HIV-1 (strain IIIB). It contained a reverse transcriptase (RT) activity of 1.5 x 10^6 cpm/ml with an infectivity of 3 x 10^6 TCID50 (tissue culture infectious dose 50) equivalents for lymphocytes, determined as previously described (14). 1 ml of purified, high-titer stock of HIV-1 was first heat inactivated of infectious virus at 56°C for 45 min, and then added to CD34+ cells for 2 h at 37°C. Control (mock-treated) cultures were run in parallel by challenging CD34+ cells with 1 ml of the supernatant of uninfected H9 lymphoblastoid T cells cultured under optimal conditions. After virus adsorption, the cells were plated in liquid or semisolid cultures. The absence of infectious virus after heat inactivation was checked by adding HIV-1 IIIB to permissive T lymphoblastoid H9 and Jurkat T cell lines or PHA-stimulated PBMC. In some experiments, CD34+ cells were treated with heat-inactivated HIV-1 plus increasing concentrations (1-100 ng/ml) of purified TGF-β1.

In experiments with recombinant env proteins, several doses (0 ng-10 μg) of baculovirus-derived HIV-1 gp120 (ABT, Cambridge, MA) were added to cells for 1 h at 4°C followed by 30 min at 4°C with 20 μl rabbit anti-gp120 (ABT) serum before plating. To control for nonspecific protein effects, we performed experiments with baculovirus-derived recombinant p24 (ABT) murine IgG, human IgG followed by 20 μl of rabbit anti-p24 (ABT), rabbit anti-mouse IgG, or rabbit anti-human IgG antiserum, respectively. Normal rabbit serum was also included as additional control.

Serum-free Suspension Cultures. To eliminate the influence of TGF-β1 contained in serum or plasma (21), purified CD34+ cells were resuspended in serum-free medium (IMDM containing 10-4 M BSA-adsorbed cholesterol and nucleosides, 10 μg/ml each, 0.5% BSA, 10 μg/ml insulin, 2% 200 μg/ml iron-saturated transferrin, 5 x 10-5 M 2-β-ME) containing IL-3 (0.4 ng/ml) and SCF (40 ng/ml). 50,000 cells/well were incubated in 48-well flat-bottom tissue culture plates (Nunc, Roskilde, Denmark) in 0.2 ml of medium at 37°C in a water-saturated atmosphere of 5% CO2 for the next 15 d. Using trypsin blue dye exclusion, the number of viable cells was determined over this 15-d period.

To minimize the influence of possible endotoxin contaminations, all the experimental procedures were performed in endotoxin-free plastic ware. According to the manufacturer’s information, the levels of endotoxin contamination in the cytokine preparations were <0.13 endotoxin U/ml by the Limulus assay (E-Toxate; Sigma Chemical Co.; limit of detection, 0.06 EU/ml).

Cell Cycle Analysis and 3HThymidine Incorporation Assay. At different time points, cells were harvested from liquid culture,
fixed in 70% ethanol for 1 h at 4°C, and then incubated with 20 μg/ml of RNase for 30 min at 37°C. Nucleic DNA was stained with 50 μg/ml propidium iodide (PI; Sigma Chemical Co.) and allowed to equilibrate for 10 min in the dark before being analyzed as described (22). Fluorescence analysis of individual nuclei was performed by the use of a FACScan flow cytometer equipped with an argon–ion laser (488-nm wavelength, 100-mW light output) and lysis II software (Becton Dickinson & Co.). The fluorescence intensity from cell nuclei stained with PI is proportional to the cellular DNA content.

For the [3H]thymidine incorporation assay, cells were counted at different time points of liquid culture, and then seeded at 50,000/100 μl in 96-well flat bottom tissue culture plates (Nunc). 1 μCi [3H]thymidine (6.7 Ci/mmol; DuPont New England Nuclear, Boston, MA) was added to each well for 4 h of incubation. Radioactivity incorporated into DNA was measured by liquid scintillation counting.

**Detection of TGF-β1 mRNA.** Total RNA was isolated from ~1 × 10^6 enriched CD34+ cells by using RNAzol B (Biotecx Texas, Houston, TX), according to the manufacturer’s instructions, and resuspended in 10 μl of diethylpyrocarbonate-treated water. Reverse transcription was performed for 10 min at room temperature and for 60 min at 42°C on 1 μg of RNA (equivalent to 5 × 10^5 cells) in 20 μl of a reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 0.1% gelatin, 100 μM random hexamer primers, 20 U of plasmid RNAse inhibitor (Boehringer Mannheim, Postfach, Germany), 100 U of RT (Perkin-Elmer Cetus Instruments, Norwalk, CT), and 1 mM of dATP, dCTP, dGTP, and dTTP. Each cDNA sample was then used as template for the PCR assay. The PCR mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.1% gelatin, 100 μM of TGF-β1 primers, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus Instruments). The TGF-β1 primers 5'-CAGAAATACAGCAACAATTCCTGC-3' and 5'-TTGCAGTGTGTATCCGTGCTGTC-3' were prepared according to Kekow et al. (23) and define a 186-bp fragment extending from +1358 to +1544 in the TGF-β1 transcript (24). PCR reaction was performed in a 100-μl vol for 35 cycles (denaturating 1' at 94°C, annealing 1' at 55°C and extension 1'30'' at 72°C). 20 μl of the PCR products was then resolved in a 3% agarose gel. The positive control was a TGF-β1 cDNA digested with PstI (24), whereas the negative control was a TGF-β2 cDNA digested with BglII (25), each at 1,200 template copies.

**TGF-β1 Protein Determination.** Supernatants were collected from serum-free 3d CD34+ cell suspension cultures. These samples were tested for TGF-β1 activity after transient acidification: pH in the supernatants was reduced to pH 2 by the addition of 5 mol/liter HCl for 2 h and then neutralized to pH 7 with 1.4 mol/liter NaOH in 0.7 mol/liter Hepes. Titers of TGF-β1 were expressed in nanograms per milliliter based on a standard curve that was generated with each set of assays by using purified human TGF-β1 (R&D Systems, Inc.). The total amount of TGF-β1 in CD34+ cell culture supernatant was determined by antibody neutralization. The bioassay on CCL64 mink lung epithelial cells (26) was performed as described previously (27). Briefly, 10^6 CCL64 cells/well were seeded in 0.2 ml of serum-free medium in 96-well flat-bottom tissue culture plates (Nunc). Serial concentrations of purified TGF-β1 or CD34+ cell culture supernatants were added to CCL64 cells in appropriate dilutions in the absence or presence of 20 μl of anti-TGF-β1 serum. Cultures were incubated at 37°C for 24 h, and 1 μCi [3H]thymidine (DuPont) was added to each well during the final 4 h of incubation. Radioactivity was measured by liquid scintillation counting.
lying the inhibitory effect of HIV-1 virions or envelope gp120 on PB hematopoietic progenitors, a serum-free liquid suspension assay was devised to monitor the survival of CD34+ cells. Typically, 50,000 cells, in a final volume of 0.5 ml, were added per well to 48-well flat-bottom tissue culture plates containing SCF (40 ng/ml) plus IL-3 (0.4 ng/ml). As can be seen in Fig. 2 A, these culture conditions allowed for the survival and minimal proliferation of CD34+ cells during the next 15 d at 37°C, at which time they plateaued at only a three- to fourfold amplification of starting cells. Of note, >60% of the 15-d cell population was still CD34+. At this time point, no significant differences in the phenotypic expression of CD34+ antigen were observed between HIV-1-mock-treated and treated groups (data not shown). In the presence of HIV-1 or cross-linked gp120, a significant (p < 0.01) inhibition of the total number of viable cells was observed from days 6-9 onward. Consistently, a progressive inhibition of DNA synthesis with lower levels of [3H]thymidine incorporation was seen in HIV-1/gp120-treated cultures from day 3 onward (Fig. 2 B). Analysis of the cell cycle performed by flow cytometry after PI staining showed that >60% of control cells were in G0/G1 at any time point considered with a background (<2%) of subdiploid DNA (Table 1). HIV-1 and gp120 were used at the same concentrations reported in the legend to Fig. 1. Data represent the mean ± SD of four to six separate experiments performed in duplicate.

We then set out to determine whether the inhibitory effect of HIV-1 glycoproteins on the survival and clonal growth of hematopoietic progenitors could be mediated by TGF-β1, a pleiotropic cytokine that plays an important physiological role in the negative regulation of hematopoiesis (21, 29). Several lines of evidence suggested that TGF-β1 could be the mediator of the HIV-1/gp120-induced inhibition. In fact, (a) an autocrine production of TGF-β1 by hematopoietic progenitor cells has been dem-
Table 1. Cell Cycle Analysis of PB CD34⁺ Cells Performed at Different Time Points of Suspension Culture

| Sampling time | Cell cycle composition (%) | Cells with subdiploid DNA (%) |
|---------------|---------------------------|-------------------------------|
|               | S             | G2/M         | G0/G1        | % of cells | % | % |
| 3 d           |               |              |              |            |   |   |
| Control       | 23            | 8            | 69           | 2          |   |   |
| HIV-1         | 11            | 4            | 85           | 6          |   |   |
| HIV-1 + CD4  | 21            | 9            | 70           | 2          |   |   |
| Cross-linked gp120 | 9  | 3            | 88           | 7          |   |   |
| Cross-linked p24 | 25 | 9            | 66           | 1          |   |   |
| 6 d           |               |              |              |            |   |   |
| Control       | 26            | 9            | 65           | 1          |   |   |
| HIV-1         | 7             | 3            | 91           | 9          |   |   |
| HIV-1 + CD4  | 24            | 10           | 66           | 3          |   |   |
| Cross-linked gp120 | 7  | 3            | 90           | 10         |   |   |
| Cross-linked p24 | 28 | 10           | 62           | 2          |   |   |
| 12 d          |               |              |              |            |   |   |
| Control       | 29            | 9            | 62           | 2          |   |   |
| HIV-1         | 3             | 2            | 95           | 11         |   |   |
| HIV-1 + CD4  | 26            | 8            | 66           | 1          |   |   |
| Cross-linked gp120 | 4  | 3            | 93           | 12         |   |   |
| Cross-linked p24 | 26 | 10           | 64           | 3          |   |   |

Data represent the means of three separate experiments performed in duplicate. Standard deviations were within 9% of the mean.

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or cross-linked p24. The addition of soluble CD4 completely blocked the biological activity of TGF-β1 recovered in HIV-1–treated CD34+ cells. Interestingly, a suppressive effect on the colony formation, similar to that previously found in the presence of either HIV-1 virions or cross-linked gp120 (Fig. 1 B), was also observed in CD34+ cell cultures supplemented with low concentrations (1–10 ng/ml) of purified TGF-β1 (Fig. 7 A). These concentrations were comparable to those found in the culture supernatant of HIV-1/gp120–treated CD34+ cells. Together, these data further suggest that minimal amounts of endogenous TGF-β1 released by CD34+ cells can account for the inhibitory activity of HIV-1/gp120.

Since elevated levels of circulating TGF-β1 have been documented in HIV-1–seropositive subjects (34), and TGF-β1 is able to trigger its own production (35), we next evaluated the effect of purified TGF-β1 in combination with
Figure 6. (A) TGF-β1 mRNA expression in CD34+ cells. (A) Lane M, molecular weight markers; lane 1, TGF-β2 cDNA (negative control); lane 2, TGF-β1 cDNA (positive control); lane 3, CD34+ cells treated with H9 uninfected cell culture supernatant; lane 4, H2O (negative control); lane 5, CD34+ cells treated with heat-inactivated HIV-1; lane 6, CD34+ cells treated with heat-inactivated HIV-1 plus soluble CD4. (B) Quantitative evaluation of the amount of TGF-β1 released in culture supernatants by CD34+ cells 3 d after exposure to H9 uninfected cell culture supernatant (control), heat-inactivated HIV-1, cross-linked gp120, heat-inactivated HIV-1 plus soluble CD4, and cross-linked p24 used at the same concentrations reported in the legend to Fig. 1. A representative of three separate experiments is shown.

Figure 7. Suppressive effect of increasing concentrations of TGF-β1 alone (A) or in combination with heat-inactivated HIV-1 (B) on the colony size of different types of progenitors in semisolid cultures. Data are reported as mean ± SD of three to five separate experiments performed in duplicate.

In a last group of experiments, we investigated the effect of the addition of antisense TGF-β1 oligonucleotides or anti-TGF-β1 serum to CD34+ cells purified from the PB of three HIV-1-seropositive individuals, whose hematologic parameters are shown in Table 2. Of note, the presence of HIV-1 infection in enriched CD34+ cells was preliminarily excluded by gag DNA PCR, performed as described previously (7). A substantial increase in the num-

Table 2. Hematological Features of HIV-1-seropositive Patients

| Patient | Sex | Pit | Hb  | WBC  | CD4 | CD8 | gag DNA in CD34+ cells |
|---------|-----|-----|-----|------|-----|-----|------------------------|
|         |     | ×10⁵/liter | g/dl | ×10⁹/liter | ×10⁹/liter | ×10⁹/liter |
| 1       | M   | 76   | 13.0 | 4,250 | 128 | 497 | negative               |
| 2       | M   | 96   | 11.5 | 5,100 | 89  | 715 | negative               |
| 3       | F   | 107  | 10.6 | 3,900 | 113 | 863 | negative               |

Pit, platelets; Hb, hemoglobin; WBC, white blood cells; CD4, CD4+ T cells; CD8, CD8+ T cells.
enhancement of colony number (A) and size (B) by different types of progenitors in semisolid cultures, and total number of CD34+ cells (C) purified from three HIV-1-seropositive subjects in suspension cultures in the presence of TGF-β1 antisense oligomers or anti-TGF-β1 serum. Data are reported as means of experiments performed in duplicate.

Figure 8. Enhancement of colony number (A) and size (B) by different types of progenitors in semisolid cultures, and total number of CD34+ cells (C) purified from three HIV-1-seropositive subjects in suspension cultures in the presence of TGF-β1 antisense oligomers or anti-TGF-β1 serum. Data are reported as means of experiments performed in duplicate.
nous TGF-β1 on hematopoietic progenitors could be potentiated by combination with inhibitory cytokines, including exogenous TGF-β1, produced by infected bone marrow and PB accessory cells in response to HIV-1 infection or exposure to recombinant proteins (15, 23, 27, 42–44).

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