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

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/gp120 inhibitory effect on CD34+ hematopoietic progenitors. To do this, we studied the effect of anti-TGF-β1 serum or TGF-β1 antisense oligomers on the survival and clonogenic capacity of CD34+ cells, purified from the peripheral blood of healthy donors and HIV-1–seropositive carriers, in both serum-free suspension and semisolid assays.

Materials and Methods

Growth Factors, Antibodies, and Oligodeoxynucleotides. rIL-3 and stem cell factor (rSCF), 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 (10^4 neutralizing units/ml; Biosource, Camarillo, CA) and rabbit anti-TNF-α (Genzyme Corp.) polyclonal sera were used. In preliminary experiments, 20 μl of anti-TGF-β1 serum could completely neutralize 100 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 Hatzfeld et al. (19). The sequence of the phosphorothioate oligonucleotides are as follows: TGF-β1 antisense, 5'-CCCGGAGGGCGGCATGGGGGA-3'; TGF-β1 sense, 5'-TCCCCCATGCCGCCCTCCGGG-3'; TGF-β1 missense, 5'-GGCGAGCGAGTGAGCGCGCGG-3'.

Isolation of CD34+ Progenitor Cells from Peripheral Blood. Informed consent for the study was obtained according to the Helsinki declaration of 1975 from 20 healthy donors and 3 HIV-1–seropositive subjects. Mononuclear cells were isolated from leukapheresis 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 × 10^6 cells/tube. 50 μl of the following mAbs were added to each tube: anti-CD2, anti-CD3, anti-CD8, anti-CD11, anti-CD14, anti-CD19, anti-CD20 (Becton Dickinson & Co., San Jose, CA) in the presence of 0.5% BSA (fraction V Chon; Sigma Chemical Co., St. Louis, MO). After two washings, 100 × 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 × 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 averaged ~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 10^4 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.

Virus Stock and Recombinant Viral Proteins. Virus stock was represented by the supernatant of H9 lymphoblastoid T cells cultured at optimal cell density (0.5–1.5 × 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 × 10^6 cpm/ml with an infectivity of 3 × 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 59°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 (10 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 antisera, 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 × 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% CO$_2$ for the next 15 d. Using trypan 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 [³H]Thymidine 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 × 10⁵ 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 × 10⁴ 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 pM random hexamer primers, 20 U of placental 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 pM of TGF-β1 primers, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus Instruments). The TGF-β1 primers 5′-CAGAAATACAGCAACAATTCCTGC-3′ and 5′-TTGC-AGTGTGTTATCCGTGCTGTC-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 (denaturing 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 BamH1 (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, 10⁵ 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.

Assay for Clonogenic Cells and Scoring Criteria. Colony assays for erythroid (BFU-E), granulocyte/macrophage (CFU-GM), and megakaryocyte (BFU-meg and CFU-meg) progenitors were performed in serum-free fibrin clot cultures as previously described (28). Briefly, 1,000 CD34⁺ cells were seeded in IMDM supplemented with 300 μg/ml iron saturated transferrin, 3 mg BSA, 280 μg/ml CaCl₂; 10⁻⁴ M BSA-adsorbed cholesterol, 20 μg L-asparagine, 1.7 × 10⁶ M insulin, nucleosides (10 μg/ml each), 0.1 ml of 0.2% (wt/vol) purified fibrinogen resuspended in PBS, and 0.1 ml of 0.2 U/ml purified human thrombin (95%) in PBS. All reagents, except fibrinogen (provided by Kabi AB, Stockholm, Sweden), were purchased from Sigma Chemical Co.

For the identification of megakaryocyte aggregates, after 12 (CFU-meg) and 21 (BFU-meg) d of culture, fibrin clots were fixed in situ with methanol–acetone (1:3) for 20 min, washed with PBS and double-distilled water, air dried, and stored at −20°C until immunofluorescence staining was performed. Megakaryocyte colonies were composed of cells intensively fluorescent to anti-CD41W (Becton Dickinson & Co.) mAb directed against the glycoprotein IIb/IIIa complex. CFU-meg were always unifocal and composed of 3–50 megakaryocytic cells/colony, whereas BFU-meg were mainly plurifocal (from 2 to 7 foci of development) and usually composed of >50 cells/colony.

Pure erythroid and mixed granulocyte/macrophage colonies were identified in situ according to standard morphological criteria at the day of maximal growth (12 d). Whereas BFU-E were readily detectable for the presence of hemoglobin, for the identification of CFU-GM, fibrin clots were fixed and stained with Wright-Giemsa.
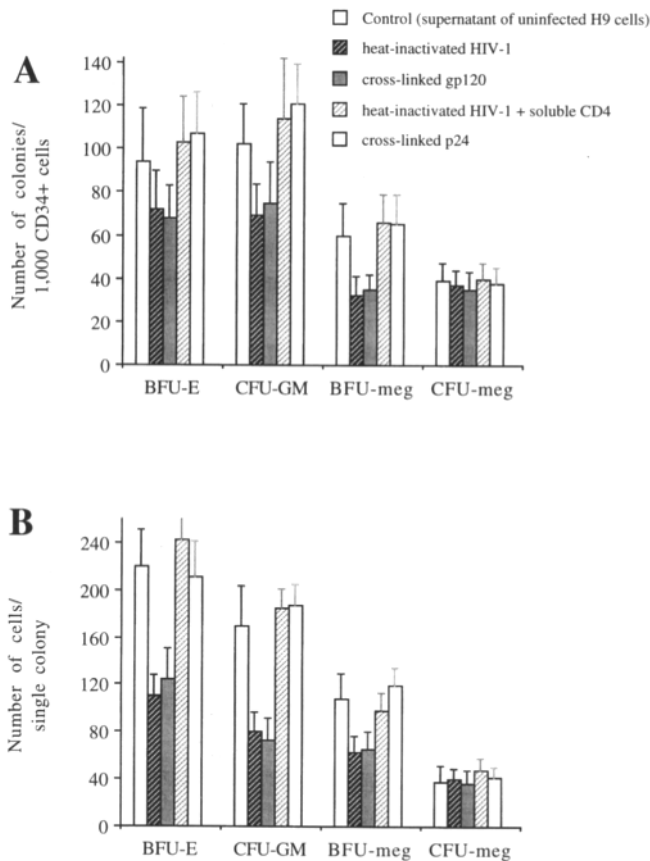
Statistical Analysis. The mean of the values ± SD for different experiments are shown in the Figures and Tables. Significant differences between treatment groups were determined by using the two-tailed Student's *t* test applied for unpaired data.

Results and Discussion

Purified peripheral blood (PB) CD34⁺ cells were challenged with either high titers of heat-inactivated HIV-1 (IIIB strain) or cross-linked gp120, and then seeded in a serum-free fibrin clot semisolid assay. We tested these reagents over a wide range of concentrations, and the results, shown in Fig. 1, are those obtained with concentrations that gave the most pronounced effects on colony formation.
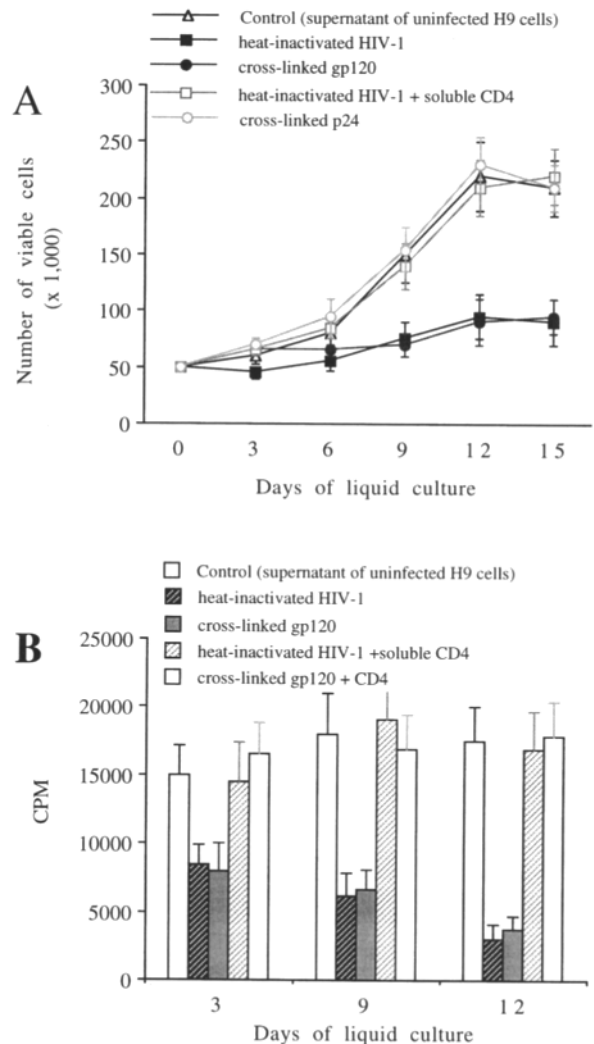
The overall plating efficiency of the cells in control cultures containing optimal concentrations of IL-3, SCF, and Ep was only partially reduced in the presence of HIV-1 virions or viral glycoproteins. In fact, the difference with control cell cultures was statistically significant ($p < 0.05$) only for BFU-meg (Fig. 1 *A*). On the other hand, cell cultures treated with HIV-1 or cross-linked gp120 showed a substantial ($p < 0.01$) decrease in the size (number of cells/colony) of all colonies except CFU-meg that were unaffected (Fig. 1 *B*). Of note, such inhibitory activity could be completely reversed by pretreatment of HIV-1 or gp120 with soluble CD4, suggesting the existence of specific interactions between envelope gp120 and the CD4 antigen expressed at low level by CD34⁺ hematopoietic progenitors (13, 17, 18).

As a first step toward elucidating the mechanisms under-

A

Number of colonies/ 1,000 CD34+ cells

□ Control (supernatant of uninfected H9 cells)
▨ heat-inactivated HIV-1
▬ cross-linked gp120
▨ heat-inactivated HIV-1 + soluble CD4
□ cross-linked p24

BFU-E CFU-GM BFU-meg CFU-meg

B

Number of cells/ single colony

BFU-E CFU-GM BFU-meg CFU-meg

Figure 1. Inhibitory activity of heat-inactivated HIV-1 (3 × 10⁶ TCID50) and cross-linked gp120 (5 μg/ml + 20 μl of anti-gp120 serum) on the colony number (A) and size of different types of progenitors (B) in semisolid cultures. Data are reported as mean ± SD of five separate experiments performed in duplicate.

A

Number of viable cells (x 1,000)

—△— Control (supernatant of uninfected H9 cells)
—■— heat-inactivated HIV-1
—●— cross-linked gp120
—□— heat-inactivated HIV-1 + soluble CD4
—○— cross-linked p24

Days of liquid culture

B

□ Control (supernatant of uninfected H9 cells)
▨ heat-inactivated HIV-1
▬ cross-linked gp120
▨ heat-inactivated HIV-1 +soluble CD4
□ cross-linked gp120 + CD4

CPM

Days of liquid culture

Figure 2. CD34⁺ cells were allowed to grow in serum-free suspension cultures as described in Materials and Methods and total cell numbers (A) and thymidine incorporation (B) were, determined at various time points. 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.

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 or cross-linked gp120 induced a significant accumulation of cells in G0/G1 phases (80–90%) with a progressive increase of subdiploid DNA (up to 10–11%), which is considered characteristic of apoptosis. An example of such experiments is reported in Fig. 3.

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.

onstrated and proposed as an important regulatory mechanism of quiescence for stem and early hematopoietic progenitor cells (19, 20); (*b*) TGF-β1 is known to decrease the total cell output in liquid culture by either arresting cell cycle progression or inducing apoptosis of hematopoietic progenitor cells (29–32); and (*c*) TGF-β1 is also able to accelerate the differentiation of early erythroid progenitors by significantly reducing the time required for the majority of CD34⁺ cells to become differentiated (33). All these properties of TGF-β1 were compatible with our present exper-

imental findings on the inhibitory activity of HIV-1 and envelope gp120.

Therefore, in the next group of experiments, PB CD34⁺ cells were first challenged with heat-inactivated HIV-1 or gp120, and then seeded in liquid suspension culture with or without 20 μl of rabbit anti-TGF-β1 neutralizing serum or control anti–mouse IgG, anti-TNF-α, or anti-IFN-α sera (Fig. 4). After 12–15 d of culture, 1.5–2-fold increase in the number of viable cells was noticed in control cultures supplemented with anti-TGF-β1 (Fig. 4 *A*). Significantly, an even greater increase (3–3.5-fold) in total cell number was found in cultures challenged with HIV-1 (Fig. 4 *B*) or cross-linked gp120 (Fig. 4 *C*) in the presence of anti-TGF-β1 serum.

To further assess the role of TGF-β1 in the HIV-1–induced impairment of hematopoietic progenitor cell growth, PB CD34⁺ cells were plated under optimal growth conditions at low cell concentration in 35-mm Petri dishes in serum-free fibrin clot semisolid assay. Addition of 8 μM of the antisense but not the sense or missense TGF-β1 significantly ($p < 0.01$) enhanced the size of BFU-E, CFU-GM, and BFU-meg (Fig. 5). Once again, the stimulatory effect of antisense TGF-β1 oligomers was greater in HIV-1–treated (Fig. 5 *B*) or gp120-treated (Fig. 5 *C*) cultures than in control (Fig. 5 *A*) cultures. Moreover, the addition of anti-TGF-β1 neutralizing serum yielded a similar enhancement ($p < 0.01$) of colony formation as achieved with addition of antisense TGF-β1. Single-cell cultures from two separate experiments gave similar results (data not shown).

To provide an additional and direct proof that TGF-β1 was produced by purified hematopoietic progenitor cells, TGF-β1 mRNA was analyzed by RT-PCR in CD34⁺ cells (Fig. 6 *A*). Expression of TGF-β1 mRNA was found in CD34⁺ cells treated with either the culture supernatant of uninfected H9 cells or heat-inactivated HIV-1. Moreover, to demonstrate that TGF-β1 protein was released in the culture supernatant by CD34⁺ cells and acted externally to the cells, a highly sensitive bioassay on the CCL64 epithelial cell line was used (Fig. 6 *B*). This group of experiments showed that significantly ($p < 0.01$) higher amounts of bioactive TGF-β1 were released in the serum-free culture supernatant by CD34⁺ cells 3 d after treatment with heat-inactivated HIV-1 or cross-linked gp120 with respect to cells treated with the supernatant of uninfected H9 cells
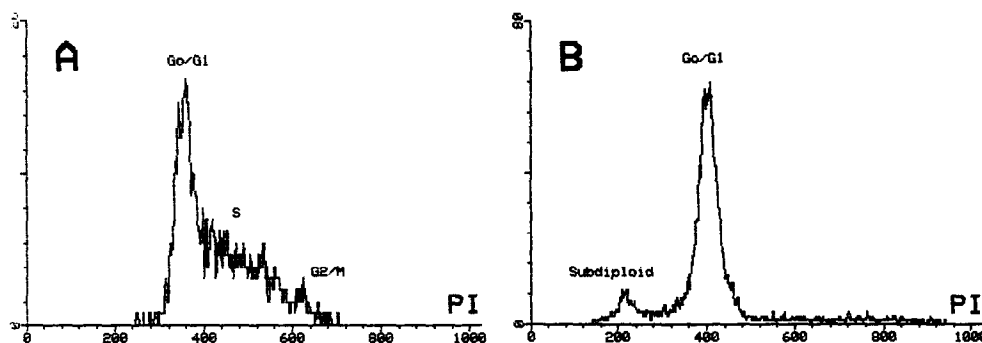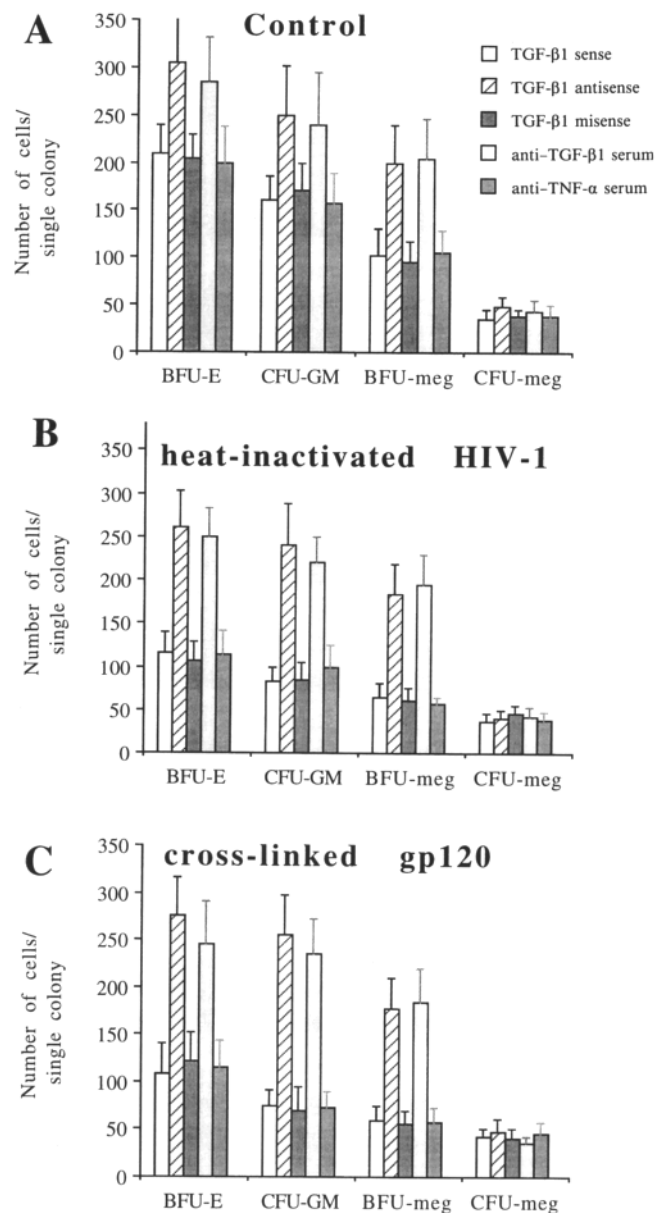

Figure 3. Cell cycle analysis of CD34⁺ cells treated with H9 uninfected cell culture supernatant (*A*; control) and heat-inactivated HIV-1 (*B*) after 12 d of suspension cultures as described in Materials and Methods. A representative of three separate experiments is shown. *x* axis, PI fluorescence. *y* axis, relative number of cells.
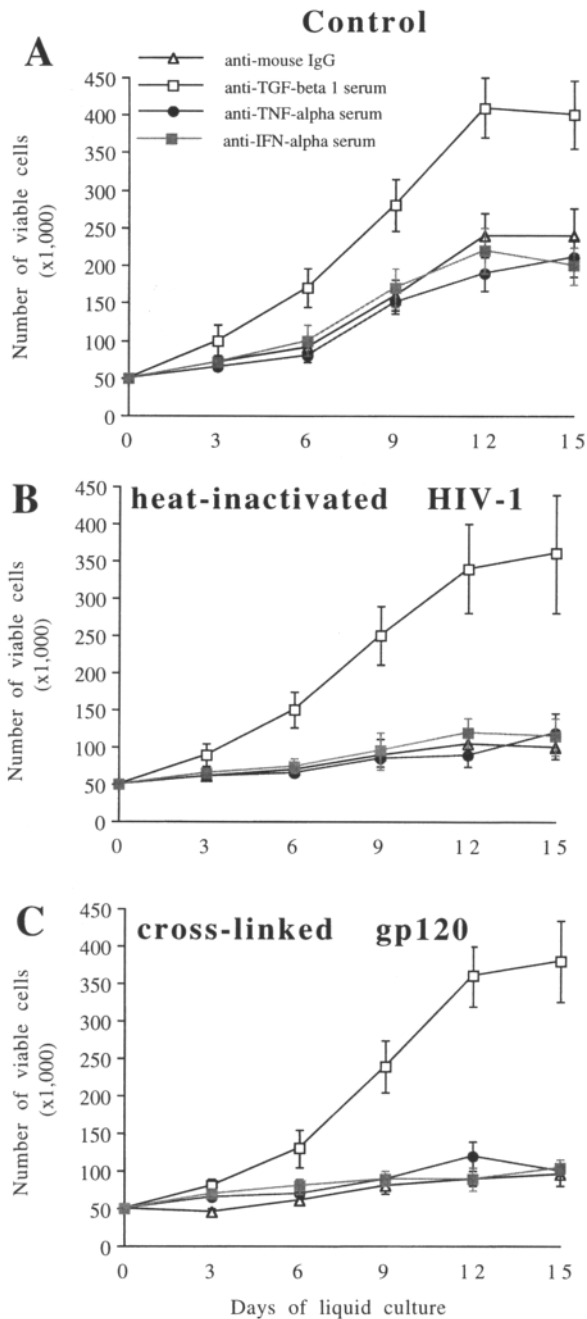
Figure 4. Enhancement of total CD34$^+$ cell numbers grown in serum-free suspension cultures in the presence of anti-TGF-β1 serum. Cells were supplemented with H9 uninfected cell culture supernatant (A; control), heat-inactivated HIV-1 (B), or cross-linked gp120 (C) using the same concentrations reported in the legend to Fig. 1. Data represent the mean ± SD of four separate experiments performed in duplicate.


Figure 5. Enhancement of colony size by different types of progenitors plated at low cell density (10^3 cells/ml) in the presence of TGF-β1 antisense oligomers or anti-TGF-β1 serum. Cells were supplemented with H9 uninfected cell culture supernatant (A; control), heat-inactivated HIV-1 (B), or cross-linked gp120 (C) using the same concentrations reported in the legend to Fig. 1. Data are reported as means ± SD of five separate experiments performed in duplicate.

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
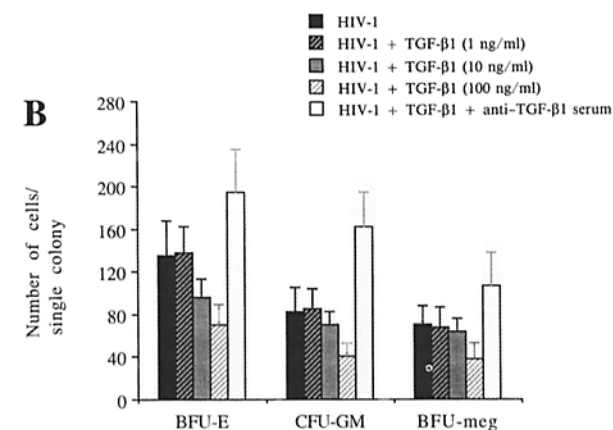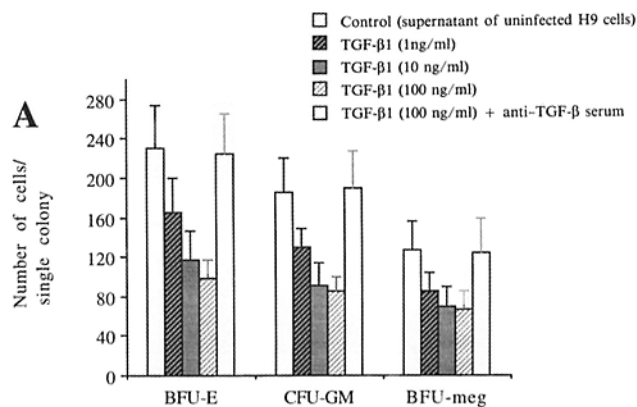
A TGF-beta 1 mRNA

M   1   2   3   4   5   6

—186 bp fragment

B TGF-beta 1 protein
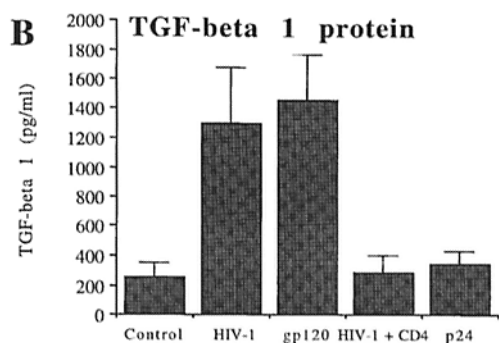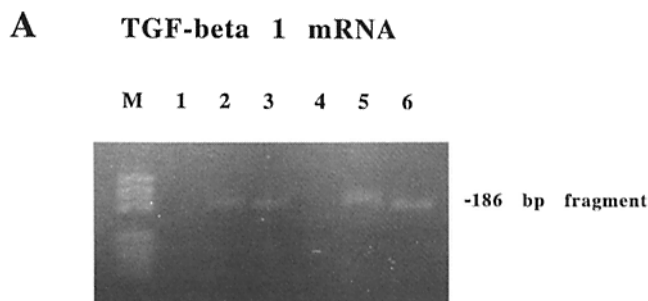
Figure 6. (A) TGF-β1 mRNA expression in CD34⁺ cells. (A) Lane M, molecular weight markers; lane 1, TGF-β2 cDNA (negative control); lane 2, TGF-β1 cDNA (positive control); lane 3, CD34⁺ cells treated with H9 uninfected cell culture supernatant; lane 4, H₂O (negative control); lane 5, CD34⁺ cells treated 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.

heat-inactivated HIV-1 (Fig. 7 B). Exposure to high concentrations of TGF-β1 (100 ng/ml) plus HIV-1 resulted in an additive inhibitory effect on CD34⁺ cell colony formation, suggesting that exogenous TGF-β1 may also participate in the upregulation of endogenous TGF-β1 in hematopoietic progenitors.

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	Plt	Hb	WBC	CD4	CD8	gag DNA in CD34⁺ cells
		× 10⁹/liter	g/dl	× 10⁶/liter	× 10⁶/liter	× 10⁶/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

Plt, platelets; *Hb*, hemoglobin; *WBC*, white blood cells; *CD4*, CD4⁺ T cells; *CD8*, CD8⁺ T cells.

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.

progenitor cells are recruited from quiescence by early-acting cytokines into a colony-stimulating responsive state that enables subsequent proliferation and differentiation (36). The entry into cell cycle, however, is regulated in a feedback loop fashion: inhibitory molecules, such as TGF-β1, limit the cycling of stem cells and consequently dampen the proliferative stimulus (21). TGF-β1 is also a potent differentiation inducer of cycling progenitors, since it may prematurely trigger terminal differentiation in primitive hematopoietic progenitor cells, thereby reducing the overall number of mature cells (33).

An HIV-1/gp120 suppression of hematopoiesis based on an indirect mechanism, such as an increased release of endogenous TGF-β1, may explain several findings reported in the literature: (*a*) the frequency of CD34$^+$ cells (number of CD34$^+$/bone marrow mononuclear cells) is not significantly reduced until very late stages of HIV-1 disease (3–9); (*b*) nevertheless, CD34$^+$ cells challenged in vivo or in vitro with HIV-1 or viral products show, at least in some studies, an impaired colony-forming ability (3–7, 12–16); and (*c*) no correlations are found between presence of viral infection, either productive or latent, and impaired colony growth of CD34$^+$ hematopoietic progenitors purified from HIV-1–seropositive subjects (3–7, 9).

Experimental evidence suggests that TGF-β1 acts on target cells by arresting or delaying cells in G0/G1 of the cell cycle (30, 31), as well as by inducing apoptosis (32). Both of these effects were observed in HIV-1/gp120–treated CD34$^+$ cells. The activity of TGF-β1 is mediated, at least in part, by maintaining the retinoblastoma growth suppressor protein in an active underphosphorylated state (37, 38) through an inhibition of the synthesis of the cyclin-dependent kinase (and/or its associated cyclin) that phosphorylates the retinoblastoma or a block of the formation of this cyclin–kinase complex (39). TGF-β1 may also inhibit cell cycling by down modulating the expression of growth factor receptors (40).

Remarkably, neither a productive nor latent infection was required to upregulate endogenous TGF-β1. Therefore, defective virions and/or free glycoprotein gp120, which are produced in abundance by infected cells (41), may be effective as infective virions in the induction of this TGF-β1–mediated inhibitory effect. The mechanism(s) by which HIV-1 or free gp120 upregulates the production and release of TGF-β1 remains to be fully elucidated. Analogous to mature myeloid cells (42, 43), purified CD34$^+$ hematopoietic progenitor cells also constitutively express TGF-β1 mRNA. However, due to the low number of CD34$^+$ cells achievable from each sample, the presence of TGF-β1 mRNA could only be evaluated by RT-PCR. This analysis did not show any significant quantitative difference in the levels of TGF-β1 mRNA between cells treated with the supernatant of H9 uninfected cultures and cells treated with heat-inactivated HIV-1. Clearly, it will be important to determine whether gp120 affects the stability of mRNAs or the translation of TGF-β1 in CD34$^+$ cells, as proposed for mature monocytes/macrophages.

Finally, our data indicate that the influence of endoge-

ber of viable cells was noticed in suspension liquid culture (Fig. 8 *A*, 4–5 fold increase after 12–15 d) as well as in the number (Fig. 8 *B*) and size (Fig. 8 *C*) of colonies in the presence of either antisense TGF-β1 oligonucleotides or anti-TGF-β1 serum.

Our results indicate that the suppressive effect induced by HIV-1/gp120 on CD34$^+$ hematopoietic progenitor cells is mediated by an upregulation of TGF-β1, endogenously produced by CD34$^+$ hematopoietic progenitors. The current model of hematopoiesis is that stem and early

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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