Human Immunodeficiency Virus Infection of Bone Marrow Endothelium Reduces Induction of Stromal Hematopoietic Growth Factors


The majority of human immunodeficiency virus (HIV)-seropositive patients develop bone marrow abnormalities associated with hematopoietic malfunction during the progression of disease. One important manifestation of HIV-associated hematopoietic dysfunction is that after myelosuppression, bone marrow recovery, a process known to be mediated in part by the production of stromal cell-derived hematopoietic growth factors, is impaired. We sought to test the hypothesis that bone marrow stromal cells infected by HIV-1 in vivo and that production of certain stromal cell-derived hematopoietic growth factors is deficient as a consequence. In this report, we demonstrate that bone marrow microvascular endothelial cells (MVEC), a key element of the stroma, are the predominant cells infected by HIV (5% to 20%) in bone marrow stromal cultures obtained from 11 consecutive HIV-seropositive patients. Although HIV-infected stromal cultures enriched for MVEC constitutively express normal levels of interleukin (IL)-4, IL-6, granulocyte (G)-colony-stimulating factor (CSF), granulocyte-macrophage (GM)-CSF, tumor necrosis factor (TNF)-\(\alpha\), transforming growth factor (TGF)-\(\beta\), and Steel factor, IL-1\(\alpha\)-induced release of IL-6 and G-CSF is significantly reduced in these cultures. These observations suggest that HIV infection of bone marrow MVEC reduces the capacity of hematopoietic stroma to respond to regulatory signals that normally augment blood cell production during periods of increased demand.

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MATERIALS AND METHODS

Isolation of endothelial cells from bone marrow aspirates. Heparinized bone marrow was aspirated from 11 HIV-seropositive patients and five normal volunteers. Six of the HIV seropositive patients had CD4 counts above 500/\(\mu\)L, and five had CD4 counts below 250/\(\mu\)L. All but one of the HIV-seropositive patients had an absolute neutrophil count greater than 1,000/\(\mu\)L, indicating that these individuals did not have severe marrow dysfunction. Three members of each HIV-seropositive group were receiving antiretroviral treatment. Informed consent was obtained from all volunteers and patients before bone marrow aspiration, and the protocol was approved by the Institutional Review Board of the Oregon Health Sciences University (Portland, OR). The clinical and hematologic characteristics of the HIV-seropositive patients are given in Table 1.

Each bone marrow aspirate was diluted 1:1 with Hanks' balanced salt solution (HBSS). Low-density cells were separated by Hypaque-Ficoll density gradient centrifugation, washed twice in HBSS, and resuspended in 3 ml serum-free Endothelial-SFM medium (GIBCO, Gaithersburg, MD). Resuspended cells were then applied to a glass bead column and incubated in a 5% CO\(_2\) incubator for 30 minutes at 37°C. The glass bead adherence step allowed preferential retention of endothelial cells along with some fibroblasts, while bone marrow progenitors, lymphocytes, macrophages, and the majority of HIV-seropositive patients to test the hypothesis that bone marrow MVEC are permissive targets for HIV-1.

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of fibroblasts were eluted from the column. The glass bead column was prepared by loosely packing a 12-cc syringe with 1.5-mm diameter glass beads (fisher Scientific, Pittsburgh, PA) up to the 6-ml mark. The glass bead column was pre-equilibrated by rinsing with warm Endothelial-SFM. Glass bead-adherent cells were recovered by gentle trypsinization and plated in an endothelial selection medium (Endothelial-SFM medium supplemented with 10% human AB serum, 25 μg/ml endothelial cell growth supplement, and 40 μg/ml heparin [all from Sigma, St. Louis, MO]) in Primaria (Becton Dickinson, San Jose, CA) 35-mm diameter tissue culture dishes. Cells grew from isolated adherent faci to yield stromal monolayers that were enriched in MVEC. Cells were fixed for immunofluorescent staining 7 to 10 days after isolation and were used for cytokine generation experiments 10 to 21 days post isolation.

**Immunofluorescent staining techniques.** For detection of p24 antigen and von Willebrand factor (vWF), stromal monolayers were fixed in 95% ethanol:5% glacial acetic acid for 10 minutes and permeabilized with 0.3% Triton-X 100 for 30 minutes at 37°C. The vWF was detected using a rabbit anti-vWF antibody (Dako, Carpinteria, CA) and a goat-anti-rabbit rhodamine conjugate (Tago, Burlingame, CA). Parafomaldehyde fixation resulted in cell shrinkage and a more diffuse pattern of vWF staining than was observed after fixation of monolayers in ethanol:glacial acetic acid.

**Focal infectivity assay for HIV.** A focal infectivity assay using CD4-positive HeLa cells (HeLa CD4 cells) as indicator cells was used to detect HIV in MVEC-enriched stromal cultures. HeLa CD4 cells were obtained from Bruce Chesebro, Rocky Mountain Laboratories, National Institute for Allergy and Infectious Diseases, Hamilton, MT. HeLa CD4 cells were plated in six-well tissue culture plates (5 × 10^5 cells per well), and 24 hours later, supernatants from MVEC-enriched stromal cultures were added. After 3 days, monolayers were fixed in methanol and stained with crystal violet to detect foci of syncytial cells. Immunos staining with an anti-p24 monoclonal antibody was used to confirm the specificity of HIV-induced syncytia.

**Cytokine assays.** To generate supernatants for cytokine assay, stromal cultures were incubated with fresh medium for 24 hours. For IL-1 induction experiments, IL-1α was added to the medium at a concentration of 0.03 ng/ml. Enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-6, granulocyte (G)-colony-stimulating factor (CSF), granulocyte-macrophage (GM)-CSF, IL-4, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and steel factor (R&D Systems Inc, Minneapolis, MN) were used to quantify these cytokines in stromal supernatants. Supernatants and standards of known concentration were added to the antibody-coated wells to generate standard curves, and results were quantified using an ELISA plate reader. The Mann-Whitney rank sum test was used to test the significance of the observed values between groups.

**RESULTS**

**Stromal cell cultures enriched in MVEC were obtained using a glass bead-adherence technique.** Culture of cells before glass bead selection yielded a dense stromal layer composed of fibroblasts, macrophages, adipocytes, and endothelial cells (Fig 1A), while cells enriched on glass beads yielded a sparser monolayer devoid of macrophages and adipocytes and enriched for endothelial cells (Fig 1B). In glass bead-selected cultures, endothelial cells comprised approximately 20% of the stromal cellular elements, as determined by expression of p24 (Fig 1C). Using this system, MVEC-enriched stroma was isolated from bone marrow samples derived from six HIV-seropositive patients with CD4 counts above 500/μL, five patients with CD4 counts below 250/μL, and five HIV-seronegative volunteers. HIV-infected stromal cells were observed in all HIV-seropositive patients' MVEC-enriched stromal cultures as determined by the presence of p24 (Fig 1D). To ascertain the infected cell type in these cultures, we used double-label immunofluorescence with antibodies directed against vWF and p24. Co-localization of both antigens identified MVEC as the infected cell type (Fig 1E and F). Although 80% of the stromal

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**Table 1. Hematologic Characteristics of the HIV-Seropositive Patients at the Time of Study**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>CD4+ T cells/μL</th>
<th>WBC × 10^9/L</th>
<th>% PMN</th>
<th>HGB (g/dL)</th>
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<tr>
<td>1</td>
<td>37/M</td>
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<td>158</td>
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<tr>
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<td>17</td>
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<tr>
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<td>161</td>
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</tr>
<tr>
<td>4</td>
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<td>70</td>
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<tr>
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Abbreviations: WBC, white blood cells; PMN, polymorphonuclear granulocytes; HGB, hemoglobin; NA, not available.
cultures were composed of fibroblasts. p24 staining was observed only in cells coexpressing VWF. The presence of HIV in stromal MVEC was consistently and reproducibly observed in all HIV-seropositive patients. In the stromal cultures, HIV-infected MVEC accounted for approximately 10% to 25% of the total endothelial cell fraction and 1% to 5% of the total cell population in patients with CD4 counts below 250/µL. The frequency of HIV-infected MVEC in patients with CD4 counts above 500/µL was decreased approximately 10-fold. To confirm the detection of HIV infection by immunoﬂuorescence, supernatants from MVEC-enriched stromal cultures derived from three of the HIV patients with CD4 counts below 500/µL were assayed for infectious virus using a focal infectivity assay on HeLa CD4 cells.33 HIV-speciﬁc foci were detected in HeLa CD4 cell monolayers exposed to supernatants from all three patient cultures. No foci were detected in HeLa CD4 cells exposed to supernatants from control cultures.

The CD34 antigen that is expressed by most hematopoietic progenitor cells is also expressed by bone marrow stromal cells.34 CD34 has been detected on vascular endothelium from a variety of different anatomic sites35-37 and recently on bone marrow MVEC.38 Examination of MVEC in enriched stromal cultures by double-labeling for CD34 and vWF (Fig 1G and H) revealed that a subpopulation of endothelial cells expressed the CD34 antigen. The degree of CD34+ expression was dependent on the age and passage number of the culture, but CD34+ MVEC were consistently vWF-positive.

In vitro HIV infection of stromal elements has been shown to impair hematopoiesis by altering production of growth factors.39-43 To directly address this issue with naturally infected cells, the production of hematopoietic growth factors was examined in HIV-infected MVEC-enriched stromal cultures. Stromal cultures derived from the 11 HIV-seropositive patients and the ﬁve HIV-seronegative volunteers were measured for the constitutive production of IL-4, IL-6, G-CSF, GM-CSF, TNF-α, TGF-β, and steel factor. Comparison of constitutive release of these seven cytokines did not reveal a signiﬁcant difference between HIV-negative and HIV-positive stroma (data not shown). However, when MVEC-enriched stromal cultures were exposed to IL-1α, a signiﬁcant reduction in the IL-1α–induced release of G-CSF and IL-6 was observed in the stromal cultures from HIV-seropositive patients relative to HIV-negative control cultures (Fig 2A through C). G-CSF was decreased from a mean of 5,197 pg/mL to 1,916 pg/mL and 1,485 pg/mL in HIV-seropositive individuals with CD4 counts below 250/µL and above 500/µL, respectively (Fig 2A). IL-6 production was reduced from a mean of 89,900 pg/mL to 40,900 pg/mL and 48,100 pg/mL in patients with CD4 counts below 250/µL and above 500/µL, respectively (Fig 2B). The levels of all the other cytokines tested were not signiﬁcantly different. GM-CSF levels are shown as a representative example (Fig 2C). These results suggest that the inability of the HIV-infected stroma to produce hematopoietic growth factors in response to IL-1α is selective and occurs early in the disease process.

**DISCUSSION**

The current studies were designed to investigate the role of bone marrow endothelial cells in inﬂuencing stromal accessory function in patients with AIDS. We have developed conditions to allow culture of bone marrow-derived stromal cells that are enriched for MVEC and depleted of macrophages. Using this system, we have determined that bone marrow MVEC derived from every HIV-seropositive patient tested were productively infected by HIV-1. In addition, we have identiﬁed a functional defect in stroma harboring HIV-infected MVEC whereby these cultures produce signiﬁcantly decreased levels of IL-6 and G-CSF in response to an IL-1α induction signal.

Several lines of evidence suggest that HIV plays a direct role in the development of peripheral blood cytopenias in patients with AIDS by affecting the proliferation and/or survival of hematopoietic progenitor cells. While the majority of studies have focused on the issue of HIV infection of CD34+ progenitor cells, a role for infection of the bone marrow stromal compartment has also been proposed. Bone marrow stromal cells comprise an adherent cell population that provides the extracellular matrix and soluble growth factors required for the proliferation and maturation of hematopoietic progenitors.24,25 It is conceivable, therefore, that infection of stromal cells could alter the hematopoietic microenvironment and, in turn, affect the development of uninfected progenitor cells.

To our knowledge, this is the ﬁrst report describing HIV infection of bone marrow MVEC. Previous attempts to locate a stromal cell target for HIV may not have identiﬁed MVEC as a permissive cell type because of the culture conditions used. Under standard conditions for long-term stromal cell culture, ﬁbroblasts rapidly become the dominant cell type. Our isolation and culture protocols enrich for MVEC, thus allowing their outgrowth in vitro and their identiﬁcation as an HIV target. We have previously reported that HIV can be detected in endothelial cells in the brains of patients with AIDS. Because of the nature of the anatomic site, brain tissue can only be examined at autopsy. We have, however, been able to conﬁrm the signiﬁcance of these ﬁndings by infecting cultured brain MVEC in vitro.26,27 HIV infection of cultured endothelial cells derived from other anatomic sites has also been described.30-32 The observation that bone marrow MVEC derived from HIV-seropositive patients are infected by HIV in vivo conﬁrms the prediction that MVEC from certain tissue sites are a natural cellular target for HIV.

We sought to investigate whether HIV infection of bone marrow MVEC was associated with a functional defect in the stroma harboring the infected cells. HIV infection of MVEC in the stroma was associated with suppression of induced but not constitutive expression of IL-6 and G-CSF. While the biologic activities of IL-6 and G-CSF are broad,4 both are known to inﬂuence the survival and/or replication of pluripotent and committed hematopoietic progenitor cells.46,47 The fact that abnormal cytokine production was observed in patients with CD4 counts above 500/µL as well as in patients with CD4 counts below 250/µL suggests that the MVEC-mediated defect occurs early in the disease course.

We have examined hematopoietic growth factor production in a coculture system comprising a mixture of endothelial cells and ﬁbroblasts. We believe that this is an appropriate system because in vivo the stromal compartment is

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Fig 2. IL-1α–induced cytokine expression is inhibited in bone marrow MVEC-enriched stroma infected by HIV. Confluent MVEC-enriched stromal cultures were exposed to 0.03 ng/mL IL-1α for 24 hours. Cytokines in conditioned media were quantified (duplicate samples) by ELISA. Control cultures (normal) were established from bone marrow samples from five normal volunteers. HIV-1–infected cultures (HIV-1+) were established from bone marrow samples from 11 HIV-1–seropositive patients, six of whom had CD4+ counts above 500/μL, and five of whom had CD4 counts below 250/μL. The 50th percentile (horizontal line within the shaded box), 25th to 75th percentile (the vertical dimension of each shaded box), and 90th percentile (vertical line) are shown for each group. The Mann-Whitney rank sum test was used to test the significance of the observed values between groups. IL-1α–induced synthesis or release of G-CSF (A) and IL-6 (B) was significantly impaired by HIV-infected MVEC stromal cultures. Induced GM-CSF expression was unaffected by HIV infection (C). Expression of TNF-α, IL-4, steel factor, and TGF-β was not affected by HIV infection (not shown).

a heterologous cell population where bidirectional cell-cell interactions regulate the cytokine profile of the hematopoietic microenvironment. Because our cultures are composed of mixed cell types, we are unable to determine whether defective cytokine production originates from the HIV-infected MVEC themselves or whether these MVEC globally influence uninfected stromal cells in the coculture. The fact that the cytokine defect was similar in both patient groups despite the difference in frequency of infected MVEC suggests that the mechanism is more complex than merely a cytokine gene expression defect occurring in an infected endothelial cell. For example, MVEC may not provide synergistic or inductive cytokines to other accessory stromal cells, or may produce an inhibitor of stromal accessory cell function. Whatever the exact mechanism, it is significant that mixed stromal populations harboring HIV-infected MVEC produce significantly less IL-6 and G-CSF than HIV-negative stromal cell cultures in response to an inductive stimulus. Because HIV infection compromises hematopoietic accessory cell function, the consequences of the defect would necessarily extend to the progenitor cell compartment. In vivo, this phenomenon could have a significant impact on the ability of the hematopoietic system to respond to inflammatory stimuli or to recover from myelosuppression.

The reduction of IL-1α–induced G-CSF and IL-6 in HIV-infected stroma is congruent with clinical observations in
HIV-seropositive patients that steady-state bone marrow function is normal, while bone marrow regeneration mediated by hematopoietic growth factors is retarded.\textsuperscript{3} Our findings are supportive of a model in which HIV-1-infected MVEC reduce the capacity of the hematopoietic microenvironment to respond to cytokine signals that augment blood cell production. Finally, bone marrow MVEC may also serve as an important reservoir of HIV-1, which uniquely positions these cells to pass virus to developing lymphoid and myeloid cells in the marrow.

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