Human Immunodeficiency Virus Infection of Human Bone Marrow Stromal Fibroblasts

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The human immunodeficiency virus (HIV) preferentially infects CD4 positive T cells and monocytes. Other human cell types have been reported to be infectable with HIV, including cells of mesenchymal origin. In this report, we show that both primary human bone marrow stromal fibroblasts and an immortalized human stromal fibroblast line are susceptible to HIV infection. These cells are capable of passing HIV to cells of lymphoid or myeloid lineage, and may thereby act as a reservoir of virus. This in vitro system may be a useful model for assessing the pathobiology of hematopoietic dysfunction in AIDS patients.

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Methods

Cells. BL is an immortalized cell line derived from adherent normal human bone marrow cells exposed to an amphotropic, murine leukemia-derived retroviral vector containing the SV40 large T antigen and the bacterial neomycin resistance gene. This cell line was isolated based on its resistance to the antibiotic G418 (GIBCO, Grand Island, NY) and its ability to sustain human bone marrow colony growth in long-term culture. The cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM; GIBCO) supplemented with 20% fetal bovine serum (FBS; GIBCO), 100 U/mL penicillin and 100 µg/mL streptomycin (P/S), and 2 mmol/L L-glutamine.

Virus stocks. Viral stocks of HIV-1 (gift of R. Gallo, NIH) and HIV-2 (gift of J. Chermann, Institute Pasteur, Paris, France) were prepared by a modification of the shaking method previously described. Briefly, H9 cells were inoculated with the appropriate HIV strain and maintained until peak level of infection (approximately 9 to 10 days). Infected cells were then washed twice with serum-free media, resuspended in one-tenth volume of serum-free media, and vigorously shaken for 2 minutes. The supernatant was收取.

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filtered through a 0.45 micron filter (Nalgene, Rochester, NY), mixed with an equal volume of FBS, and stored at -70°C until use. Viral titers were quantitated by reverse transcriptase activity (RT) or HIV p24 antigen level (Abbott Laboratories, Chicago, IL). The stock of HIV-1IB had an RT level of 2.3 x 10^6 cpm/mL, and the stock of HIV-2 had an RT level of 2.0 x 10^6 cpm/mL.

The monocytotropic strains HIV-1Ba.L (gift of M. Popovic, National Cancer Institute [NCI]) and HIV-1Kp933 (derived from bone marrow macrophages from an HIV-infected patient) were produced by the method of Perno et al. Briefly, adherent macrophages were cultured for 7 days in RPMI and 10% normal human serum and inoculated with virus. Cultures were monitored for RT and HIV p24 peak virus production (approximately 15 days after inoculation). Strain HIV-1Ba.L had an RT level of 0.8 x 10^9, and strain HIV-1Kp933 had a p24 antigen level of 3 x 10^9 pg/mL.

Cell characterization. Cells were grown on tissue culture chamber slides (LabTech, Naperville, IL) and evaluated by indirect immunofluorescence using antibodies directed against vimentin (Labsystems, Research Triangle Park, NC), cytokeratin (Labsystems) and von Willebrand’s factor (Dakopath, Carpenteria, CA). Flow cytometric analysis (Becton-Dickinson, Mountain View, CA) was done using anti-Leu-3a monoclonal antibody (Becton-Dickinson) directed against the CD4 protein. Immunohistochemistry was done for non-specific esterase activity on cells grown on tissue culture chamber slides using a kit and according to the manufacturer’s specifications (Sigma). Polymerase chain reaction was done on cDNA for the presence of CD4, CD11b (Mo 1: a monocyte marker), and actin, as described below.

In vitro infection. BL cells were grown in T-25 flasks (Corning). When approximately 80% confluent, the cells were washed with sterile 1 x PBS and incubated with 4 mL of media containing 1 x 10^8 cpm of prophylactic virus. After 24 hours, the cells were removed with 0.05% trypsin-0.53 mmol/L EDTA (GIBCO), washed 3 x with sterile PBS, and plated onto new flasks. The procedure was repeated the following day to remove residual virus. On day 5 post-infection, the cells were trypsinized again and split 1:4 with 1 aliquot frozen, 1 aliquot co-cultivated with 8 x 10^5 H9 cells in 4 mL of medium, and 1 aliquot co-cultivated with 8 x 10^5 K1 cells to amplify the level of virus produced by the stromal cells. The co-cultivated target indicator cells were harvested 24 hours later and seeded at 2 x 10^5 cells/mL in 24-well tissue culture plates (Costar, Cambridge, MA). The adherent cells were grown to visual confluence (3 to 4 days), and before each passage, cell supernatants were collected from the stromal cells and from the 24-well tissue culture plates containing target indicator cells. The supernatants were assayed for viral infection by RT activity and p24 antigen expression. The co-cultivation experiment was repeated weekly by adding fresh target cells to the stromal cells previously carried without co-cultivation. This sequence was repeated four times to evaluate the persistence of HIV infection.

Polymerase chain reaction of DNA. DNA was extracted by a method previously described. Cells were suspended at a concentration of approximately 10^6 cells/mL in a solution containing 100 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3) and 2.5 mmol/L MgCl_2, 1% Tween, and 100 µg/mL of Proteinase K (Boehringer-Mannheim, Indianapolis, IN). Samples were digested with Proteinase K for 1 hour at 60°C, followed by heat inactivation at 95°C for 10 minutes.

Polymerase chain reaction (PCR) amplification was conducted by a previously described method. For amplification of HIV-1 sequences, gag-specific primers, SK100 and SK104, were used in a reaction cycle consisting of denaturation at 90°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, repeated 15 times. One tenth of the reaction volume was then removed and used as a template for subsequent amplification with gag-specific primers, SK38 and SK39, which were located interior to the SK100 and SK104 primers on the gag region. The second amplification cycle included denaturation at 95°C for 30 seconds, annealing for 30 seconds at 65°C, and extension of 72°C for 1 minute, repeated 15 times. The product of this reaction was then denatured by boiling and mixed with 32P end-labeled SK19 in 100 mmol/L NaCl, 15 mmol/L EDTA and incubated at 56°C for 30 minutes. The product of this liquid hybridization was electrophoresed through a 10% polyacrylamide gel and autoradiographed for 2 hours, as well as overnight.

The HIV-2 isolate, HIV-2Kp93, was analyzed using similar reaction conditions; however, primers SK100 and SK104 were used with the cycle number increased to 30 and the HIV-2 specific probe SK103 applied.

Amplification of the β-globin gene using primers PC03 and PC04 was done independently to control for DNA content.

Polymerase chain reaction of cDNA. RNA was prepared by guanidine isothiocyanate lysis and cesium chloride centrifugation. First strand cDNA was generated from total RNA using 200 U MMLV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD), 0.05 mg/mL random hexanucleotide primers (Pharmacia), 3 mmol/L MgCl_2, 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 10 mmol/L diithiothrietol (DTT), 0.5 mmol/L dNTP, and 40 U Rnase (Bethesda Research Laboratories) at 37°C for 60 minutes. One tenth of this reaction volume was used in a PCR as described above using primers for CD4 cDNA (CTGAATGATCGCGGTGACTCAAG, TGTCGATGCACCTCA-CGG), beta-actin cDNA (CTTCTGTCACATCTCTGATATTTACCGG), or CD11b cDNA (gift of Dr. Folke Schreiber, Dana Farber Cancer Institute, Boston, MA; TACCTGGAGGTGGTCCACTCAG, CATCTGTACAGCATACTCCGG). Cycles of 92°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes were repeated 30 times. The reaction product was electrophoresed through a 2% agarose gel (for CD4 and actin) or an 8% acrylamide gel (for CD11b), stained with ethidium bromide, and photographed under UV transillumination. The reactions for CD4 and actin were also analyzed by hybridization to 32P-labeled oligonucleotides specific for these cDNA (GTATCTGATCGTTCTCTGTAAAG for CD4 and GGCGGAGGGTTCTCAGTGTGAAGTACGCT for actin) under conditions outlined above at 37°C, and electrophoresed through 8% acrylamide gels before autoradiography. These experiments were repeated twice with independently prepared RNA.

RESULTS

Primary human bone marrow stromal cells and the human bone marrow stromal cell line BL were characterized by immunofluorescence and histochemistry. Both primary and immortalized marrow stromal cells were uniformly negative for cytokeratin (an epithelial marker) and von Willebrand’s factor (an endothelial marker), and uniformly positive for vimentin (a mesenchymal marker) by immunofluorescence (data not shown). EJ and HUVE cells served as controls. Primary stromal and BL cells were also uniformly negative for non-specific esterase (a macrophage marker) by histochemistry, while adherent peripheral blood mononuclear cells (PBMC) were positive. Furthermore, PCR of cDNA derived both primary stromal cells and BL were negative for the monocyte/macrophage marker CD11b (Mo 1), while PBMC were positive (Fig 1). Flow cytometry analysis of primary cells or BL cells using the anti-Leu-3A antibody failed to show evidence of surface CD4 expression compared with PBL and HeLa-T4 controls. However, using the ex-
HIV INFECTION OF BONE MARROW FIBROBLASTS

A

Fig 1. PCR analysis of cDNA for expression of CD4 and actin (A) and CD11b (B). Lane 1, no cDNA control; lane 2, primary bone marrow fibroblasts; lane 3, BL; lane 4, HUVE; lane 5, F13; lane 6, EJ; lane 7, HeLa-T4; all amplified with primers for CD4. Lane 8, primary bone marrow fibroblasts; lane 9, BL; lane 10, HUVE; lane 11, F13; lane 12, EJ; all amplified with primers for actin. (B) Lane 1, 310, 281, and 271 bp size markers from Phi X digested with Hae III; lane 2, primary bone marrow fibroblasts; lane 3, BL; lane 4, PBMC; all amplified with primers for CD11b (Mo1).

B

Extremely sensitive PCR technique, expression of CD4 RNA could be detected in primary bone marrow stromal cells, BL, human foreskin fibroblasts (F13), and HeLa-T4, but not EJ cells (Fig 1). These results show a fibroblast-like origin of both the primary stromal and BL cells, that the populations of cells studied were free of monocyte/macrophage or endothelial cell contamination, and that these cells express CD4 RNA at low levels.

Primary bone marrow stromal fibroblasts were exposed to HIV-1\textsubscript{HxB} or HIV-2\textsubscript{Rod}. After several cycles of washing, trypsinization, and replating to ensure removal of residual virus, these cells were co-cultivated with the lymphoid cell line H9 or the myeloid line KG-1 to act as indicators for the presence of low levels of virus (Fig 2). Cultures of primary stromal fibroblasts alone (Fig 3) or stromal fibroblasts co-cultivated with H9 lymphoid or KG-1 myeloid cells (Fig 4) were positive for p24 antigen after exposure to HIV-1\textsubscript{HxB} or HIV-2\textsubscript{Rod}. A representative experiment of stromal fibroblasts co-cultivated with H9 lymphoid cells is shown in Fig 4. Similar results were obtained with KG-1 myeloid cells co-cultivated with stromal fibroblasts (data not shown). The titer of virus in supernatants of HIV-2\textsubscript{Rod}-exposed primary stromal fibroblasts was higher than that of HIV-1\textsubscript{HxB}-exposed stromal fibroblasts, as measured by p24 antigen (Fig 3) and RT levels (data not shown). Primary stromal fibroblast cultures remained positive for viral antigen and the ability to pass virus to lymphoid or myeloid indicator cells 21 days post-infection with HIV-2\textsubscript{Rod} or HIV-1\textsubscript{HxB}. Analysis using PCR showed that DNA from the HIV-1\textsubscript{HxB}- or HIV-2\textsubscript{Rod}-exposed primary cells was positive for the presence of the HIV genome 4 weeks post-infection (Fig 5). In contrast, exposure of primary stromal fibroblasts to the monocytotropic strains HIV-1\textsubscript{Ba.L} or HIV-1\textsubscript{B95.1} failed to result in infection measured by either direct detection of HIV p24 antigen or RT in cell culture supernatants or detection of viral DNA by PCR (data not shown).

Identical studies done as controls on murine NIH 3T3 fibroblasts and mock-infected human bone marrow stromal fibroblasts were negative. The NIH 3T3 cell line, reported to express virus when transfected with HIV DNA,\textsuperscript{17} served as the control of non-specific viral absorption or entry into cells, while the mock-infected cultures controlled for cross-contamination of the co-cultivated H9 or KG-1 cells.

Comparable results were obtained with the immortalized marrow stromal fibroblast line, BL. This line was exposed to HIV-1\textsubscript{HxB} or HIV-2\textsubscript{Rod} using the protocol above and carried per the schema in Fig 2. After exposure to virus and repeated washing and trypsinization, BL cells were co-cultivated with H9 or KG-1 cells. BL cells alone (Fig 3B) or BL cells co-cultivated with H9 lymphoid (Fig 4) or KG-1 myeloid cells (data not shown) were positive for p24 antigen after

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p24 / RT\]

\[
\text{TRYP/ SPLIT}\]

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\text{INFECTION}\]

\[
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0 1 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40\]

Fig 2. Schema of viral infection and analysis of bone marrow stromal fibroblasts. Dots indicate day of infection, trypsinization, and cell splitting (TRYP/SPLIT), or sampling of cell supernatant for HIV p24 antigen or reverse transcriptase activity (p24/RT).
Fig 3. HIV p24 antigen levels of primary bone marrow stromal fibroblasts (A) or BL (B) after exposure to HIV-1 III ( ), HIV-2 R ( ), or mock infection ( ). The upper limit of the p24 antigen assay was 500 pg/mL; samples above that limit are represented as greater than 500 pg/mL.

exposure to HIV-2R, whereas only co-cultivated cells were positive for p24 antigen after HIV-1III exposure. Over time, the p24 antigen level of the HIV-2R-exposed BL cells declined; however, passage to indicator cells was maintained up to 4 weeks post-infection. In contrast, HIV-1III-exposed cells were unable to pass virus after 3 weeks post-infection, and DNA from these cells was negative for HIV DNA by PCR at 4 weeks post-infection (Fig 4). Controls again included NIH 3T3 fibroblasts and mock-infected BL cells. Assays for viral antigen were consistently negative on the control BL and NIH 3T3 cell lines and co-cultivated indicator cell lines (data not shown). These data demonstrate successful infection of BL by HIV-1III and HIV-2R; however, in contrast with primary marrow stromal fibroblasts, HIV-1III infection was not sustained.

Infection with HIV-1III or HIV-2R in BL cells or primary marrow stromal fibroblasts did not result in morphologic changes. Syncytia formation was not evident in BL cells throughout the experiment. At the termination of the experiment, both control and infected primary fibroblasts manifested occasional syncytia, presumably due to cell senescence. Growth rates of infected and uninfected were equivalent.

**DISCUSSION**

Individuals infected with HIV commonly manifest abnormalities in peripheral blood counts. These abnormalities have several potential etiologies, including ineffective hematopoiesis and immunologically mediated peripheral destruction of cells. Abnormal hematopoiesis may result from direct effects of HIV on the bone marrow cells and inhibition of bone marrow function by opportunistic infection or neoplasm. Folks et al have reported reverse transcriptase activity in cultures of HIV-exposed hematopoietic progenitors, suggesting that direct infection of blood cell precursors by the virus may occur.18 In this study, we addressed whether HIV infection of certain stromal cells within the bone marrow compartment could occur in vitro. Stromal fibroblasts are potentially important sources of trophic or inhibitory factors regulating the development of hematopoietic progenitors. Infection of stromal cells might result in dysregulated expression of such regulatory cytokines. Furthermore, HIV-infected bone marrow stromal mesenchymal cells could serve as a potential reservoir for the passage of virus to bone marrow progenitors. HIV infection of stromal fibroblasts could contribute to a bone marrow microenvironment incapable of maintaining normal hematopoiesis.

We and others have demonstrated successful infection by HIV of cells of mesenchymal origin.2,4 The studies reported here further extend these findings to the subset of bone marrow stromal fibroblasts. Stromal fibroblasts were negative for surface CD4 expression but unexpectedly expressed low levels of CD4 RNA (as did foreskin fibroblasts) as determined by the extremely sensitive PCR technique. These cells were susceptible to infection with either HIV-1 or HIV-2, as demonstrated by the presence of HIV DNA by PCR, the presence of viral p24 antigen and RT in the culture supernatants, and the passage of virus to indicator cell lines. The presence of CD4 message in stromal fibroblasts suggests a CD4 mediated mechanism of viral entry for these cells.

Primary stromal fibroblast infection by HIV-1 appeared to be strain-specific, as we were able to infect these cells with a lymphocytotropic isolate HIV-1III but not the monocytotropic strains HIV-1R and HIV-1R9533. The immortalized stromal fibroblast line, BL, shares phenotypic characteristics with primary stromal fibroblasts, but was unable to sustain infection by HIV-1III. The basis for this aborted infection is unclear. Both primary and immortalized stromal fibroblasts produced higher levels of viral antigen after HIV-2R infection compared with HIV-1III. The phenomena has been observed in CD4 surface antigen negative cell lines by other investigators, and evidence has been gathered suggesting that rates of transcription off of the viral LTR are not the molecular basis for this difference between HIV-1 and HIV-2.19 Our observations show that viral entry and expression initially occurred, but was lost with cell passage. A possible explanation is that viral entry was substantially less
efficient with HIV-1_{III B} than the HIV-2_{Rd} and that HIV-1_{III B} may be more cytotoxic than HIV-2_{Rd}, resulting in negative selection of the infected population.

The recent report by McKeating et al of enhanced HIV infection of fibroblasts when previously infected by cytomegalovirus supports the potential relevance of our work, particularly given the frequent coincidence of these infections in patients. These observations may have implications for extending screening of anti-viral therapies beyond systems based on lymphoid or monocytic cells to include cells of mesenchymal origin. Furthermore, studies of support of in vitro hematopoiesis by HIV-infected stromal fibroblasts, particularly regulation of production of hematopoietic growth factors by such cells and passage of virus from these cells to hematopoietic progenitor cells, may provide further insights into the pathobiology of hematopoiesis in AIDS.

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