Replication of HIV in Human Fetal Retinal Cultures and Established Pigment Epithelial Cell Lines

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The ability of the human immunodeficiency virus type 1 (HIV-1) to replicate in cells derived from ocular tissue was studied. Primary retinal cultures (containing both glial and neuronal cells) were found to support the replication of HIV upon transfection with molecularly cloned proviral DNA. In addition, established retinal pigment epithelial (RPE) cell lines also produced HIV particles upon transfection. HIV released by these cell lines was able to infect and induce characteristic cytopathic effects in T4+ cells. An indicator plasmid containing the HIV long terminal repeat sequences (LTR) linked to the chloramphenicol acetyltransferase gene showed barely detectable activity in RPE cells and was transactivated by the addition of the HIV “tat” gene. Based on these observations, direct infection of ocular tissue derived cells such as RPE, fetal retinal cells, retinoblastoma cells (Y 79, WERI), choroidal endothelial cells (Chor 55) (mix culture) and corneal fibroblasts (K61) by HIV was attempted. HIV replication in these cells was not detected by reverse transcriptase, antigen and transactivation function assays. Invest Ophthalmol Vis Sci 30:1535–1541, 1989

The human immunodeficiency virus (HIV) is the etiologic agent of the acquired immune deficiency syndrome (AIDS).1 The clinical hallmark of AIDS is immune dysfunction, resulting from the selective loss of helper/inducer T cells and is characterized by multiple opportunistic infections. HIV is a cytopathic retrovirus and replicates in the T4 (helper/inducer) lymphocyte subset and monocytes in vitro.2

HIV has been isolated from different sources, including peripheral mononuclear cells, bone marrow, lymph nodes, spleen, plasma, semen and saliva of patients with AIDS or related disorders.3 Neurologic abnormalities have also been noted in AIDS4 and the virus has been recovered from the central nervous system (CNS) tissue of these patients as well.6 Multiple ocular abnormalities—Kaposi’s sarcoma of the lid and conjunctiva, cotton-wool spots and cytomegalovirus (CMV) retinitis—have been reported in AIDS patients.8 Furthermore, HIV has been cultured from the tears, retina and corneal tissues of patients with AIDS.9 The role of HIV infection in ocular complications is not clear. We investigated the ability of the HIV genome to persist and be expressed in cells from ocular tissues. Previous studies indicated that HIV was unable to establish infection of monolayer cells due to the absence of receptor for the virus on the cell surface. However, such cells were able to support the replication of HIV11 if the receptor requirement for virus entry was overcome by transfection of cloned viral DNA into the cells. Our experiments involving primary retinal cells and established retinal pigment epithelial cell lines show that HIV replicates in these cells upon transfection of proviral DNA, but there was no evidence of direct infection of RPE, retinoblastoma cells, choroidal endothelial and corneal fibroblasts by HIV.

Materials and Methods

Cell Lines

Human retinal pigment epithelial (RPE) cell lines 0041, 0308 and 0125 were maintained in Ham’s F12 medium supplemented with 16% fetal bovine serum, 0.2 mM glutamine, 0.75% bicarbonate and 0.5% sucrose. Cell lines were established by Del Monte et al13 and used between 30 and 50 passages. Cells are epithelioid in morphology, mostly depigmented and under appropriate substrate conditions express morphologic markers of epithelial cells. Cells were subcultured every third or fourth day. Primary cultures...
of choroid (CH55) were established from a 36-year-old male and at least 50% the cells were positive for factor VIII. Cells were used in passages 6–12. Primary corneal fibroblast cultures were from a 75-year-old diabetic female. Cells used in the study were in passages 6–9. Retinoblastoma cell lines Y79 and WERI cells were maintained in Ham’s F12 and RPMI 1640 medium, respectively.

Fetal retinal cultures were initiated using the eyes obtained from spontaneously aborted first trimester fetuses by mechanical dispersion of the retina through decreasing sizes of hypodermic syringe needle (gauges 16, 18, 21). Fetal retinal cultures were maintained in the same medium as RPE.

Virus Infection

HIV-1-Zr6 virus was isolated from an AIDS patient from Zaire.14 HUT 78 and fetal retinal cells were infected with HIV-Zr6 virus (5 × 10^3 cpm reverse transcriptase activity equivalent) in the presence of polybrene (1 μg/ml) as described.14 Culture supernatants were monitored for virus production. Virus was pelleted from cell free supernatants at 35000 rpm for 1 hr in a Beckmann ultracentrifuge and the pellet was suspended in 50 μl of virus solubilization buffer (50 mM Tris pH 7.8, 100 mM NaCl and 1% Triton X-100). A 20 μl aliquot was used for reverse transcriptase assay using poly(rA)-(dT)12-18 as the template primer and Mg++ as the divalent cation. Incorporation of the radiolabeled thymidine was measured by trichloroacetic acid (5% final) precipitation of the reaction mixture in the presence of saturated sodium pyrophosphate and yeast tRNA.

HIV antigen was assayed in a solid-phase sandwich-type enzyme immunoassay (EIA) using the HTLV III antigen EIA test.15 Polyclonal antibodies to HIV were used as capture and probe antibodies, and labeled second antibody was used to identify a positive reaction. The absorbance was read at 492 nm with quantum dual wavelength spectrophotometer.

Transfection

Human RPE monolayer cultures and primary fetal retinal cultures were transfected by a modified calcium phosphate coprecipitation method.16,17 Recombinant plasmid DNA containing HIV proviral sequences14 was mixed with 0.5 ml of 2 × HEPES pH 7.05 (10 g of N-2-hydroxy ethilpiperazine-N-2 ethanesulfonic acid; [HEPES] 16 g of NaCl, 0.74 g of KCl, 0.25 g of Na2HPO4, 2H2O and 2 g of dextrose per liter). An equal volume of 0.4 M CaCl2 in 10 mM Tris (pH 7.6) and 1 mM EDTA was added. The mixture was applied to 1 × 10^6 cells without carrier DNA. After 6 hr the cells were treated with 15% glycerol for 90 sec at room temperature and maintained in regular growth medium. HUT 78 (1 × 10^5) cells were transfected with plasmid DNA by a modified DEAE-dextran method18 as a positive control. Production of virus was determined at the end of 5 days by assaying the supernatant fluid for viral associated reverse transcriptase activity.19 The construction of the HIV transactivator plasmid pTAT and pLTR-CAT has been described previously.20 The plasmid DNA was prepared by double banding in CsCl.

CAT Assay

The CAT assay was performed using the procedure described by Gorman et al.21 The cells were removed from the dish approximately 48 hr after transfection in 1 ml buffer (0.15 M NaCl, 10 mM Tris [pH 7.5] and 1.5 mM MgCl2), washed and resuspended in 100 μl of 0.25 M Tris [pH 7.8]. Cell extracts were prepared by four freeze (−70°C)–thaw (37°C) cycles followed by a brief centrifugation to remove cellular debris. The protein concentration in various cell extracts was determined and amounts of cell extract corresponding to 150 μg of protein were incubated with 14C chloramphenicol (New England Nuclear Corporation, Boston, MA), in the presence of 4 mM acetyl coenzyme A at 37°C, in a total volume of 140 μl for 60 min. The reaction was terminated by the addition of 1 ml ethyl acetate. The organic phase was dried and the material was resuspended in 30 μl of ethyl acetate, spotted onto a thin layer chromatography plate (Merck and Company, Inc.), and chromatographed in chloroform methanol (95:5). The plates were dried and exposed to Kodak XAR-2 film. The percentage conversion of chloramphenicol was determined by cutting the appropriate spots from the plate and counting them in a toluene-based scintillation fluid.

Transactivation Function Assay

Cells (1 × 10^6) were infected with three different HIV isolates (designated Zr6, HTLV III22 and CDC 45123) for 24 hr. The cells were washed and growth medium was replaced. Plasmid pLTR-CAT DNA was transfected into virus infected cells by the calcium phosphate precipitation method. At the end of 72 hr after transfection, cells were scraped and CAT assay was carried out as described.16

Results

We used a full-length biologically active molecular clone of HIV generated from a virus isolated from a...
The recombinant plasmid pZ6Neo (Fig. 1) contains the HIV genome, the neomycin gene and pBR322 sequences. The neomycin marker gene was positioned downstream of 3' LTR to use the transcription signal sequences present in the LTR.

RPE cells showed the presence of budding virions after transfection with pZ6Neo (data not shown). Dot-blot hybridization of the viral particles in the medium of the transfected cultures using a full-length HIV probe confirmed the presence of HIV. Reverse transcriptase activity in the supernatants of the transfected RPE cultures revealed low-level replication of HIV in comparison to human rhabdomyosarcoma (RD) cells and the leukemic T cell line HUT 78 (Table 1). Primary fetal retinal cells (which are a mixture of neuronal and glial cells) showed a slightly higher level of HIV replication than RPE cell lines, although after 10 days virus production was minimal in all of the cells.

Nucleotide sequence information for the Zairian and other HIV isolates revealed 29 to 31 potential glycosylation sites in the "env" gene. Since posttranslational modification (eg, glycosylation) may differ by cell type, viruses recovered from transfected RPE and fetal retinal cell cultures were analyzed for their ability to infect T4+ cell type. Viruses derived from all the RPE cell lines and the primary fetal retinal cultures were able to infect HUT 78 cells productively and induced a cytopathic effect (Fig. 2). This suggests that viral proteins are synthesized and processed in RPE/retinal cells as they are in T-lymphoid cells.

The ability of HIV LTR to function in RPE cell lines was also tested. A chimeric plasmid pLTR-CAT was used in which HIV LTR was fused to the chloramphenicol acetyltransferase gene (Fig. 3). RPE cells were transfected with pLTR-CAT or cotransfected with pLTR-CAT and the HIV transactivator gene (pTAT). HIV LTR directed expression of the CAT gene was minimal in all the cell lines (Fig. 4). Cotransfection with pTAT plasmid increased CAT activity, indicating the TAT gene product functions efficiently in RPE cells.

Since RPE cells supported the replication and transactivation function of HIV, we also checked these cells for possible direct infection by HIV. RPE cells (1 x 10^6) were incubated overnight with culture supernatants containing HIV in the presence of polybrene (1 µg/ml). We used three different HIV isolates designated HTLV III, CDC 451, and Zr6 for infections. The cells used for infection experiments are listed in Table 2. Cells were washed and the supernatants were tested for the presence of HIV particles.

There was no detectable virus in the supernatant (tested up to 15 days) by reverse transcriptase and viral antigen assays (Table 2). RPE cells, exposed to the virus and passaged three times in culture, were cocultivated with phytohemagglutinin-stimulated peripheral blood lymphocytes (PBLS). PBLS did not show positive evidence for virus production.

We have also employed another sensitive assay based on transactivation to analyze whether RPE cells are infectible by HIV. Since HIV transactivator gene (pTAT) product activates HIV LTR directed expression, we used a recombinant plasmid pLTR-CAT. Transactivation of pLTR-CAT transfected into RPE cells was assayed using a CAT reporter (Fig. 4).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Reverse transcriptase activity (cpm)</th>
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<tbody>
<tr>
<td>RPE cell lines</td>
<td></td>
</tr>
<tr>
<td>0125</td>
<td>8000</td>
</tr>
<tr>
<td>0041</td>
<td>5300</td>
</tr>
<tr>
<td>0308</td>
<td>10,500</td>
</tr>
<tr>
<td>Fetal retinal cells</td>
<td>35,000</td>
</tr>
<tr>
<td>HUT 78</td>
<td>200,000</td>
</tr>
<tr>
<td>Rhabdomyosarcoma cells (RD)</td>
<td>1,250,000</td>
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</tbody>
</table>

* Untransfected cells gave <1000 cpm of reverse transcriptase activity. Results represent an average of three experiments.
the cells after infection with HIV should provide a sensitive parameter to identify cells that are infectible by HIV. Ocular tissue-derived cells did not show transactivation of pLTR-CAT expression.

Discussion

Our study demonstrates that HIV proviral DNA can direct the synthesis of viral particles in RPE cell lines and primary fetal retinal cell cultures. HIV, like other retroviruses has LTR sequences at either end of the genome. The LTRs contain promoter and enhancer sequences which in some instances have been shown to control viral gene expression in a cell-specific manner. The HIV LTR promoter functions in T cells, B cells, macrophages and also in cells of fibroblastic origin. The ability of HIV proviral DNA to produce mature virions in fibroblasts suggest the possibility of latent or chronic infection in different cell types. Budding viral particles have been noted in Kaposi's sarcoma cells, dendritic cells and macrophages. A noncytopathic productive infection could lead to a continuous release of virus. Similar questions may now be asked of RPE and retinal neurosensory cells.

We have noted in our earlier studies that established cells of rodent and primate origin can support the replication of HIV to a different extent and also in a transient manner. Viral replication in these cells can be monitored by assaying the medium of the transfected cells for reverse transcriptase activity. Adachi et al and Levy et al used cocultivation methods to quantitate virus production in some of

Fig. 2. Infection of HUT 78 cells with virus recovered from RPE cells after transfection with proviral DNA. (A) uninfected HUT 78 cells; (B) virus infected HUT 78 cells with characteristic cytopathic effects.
Fig. 3. Construction of pLTR-CAT. HIV molecular clone (HIV-III) was cleaved with Hind III to generate a 1475-bp fragment comprising sequences upstream of 3' LTR, U3 and 77 bp in the "R" region of LTR. This fragment was inserted into the pGCAT plasmid that contains the bacterial CAT gene. Arrow indicates the direction of transcription.

Fig. 4. Expression of pLTR-CAT in RPE cells. RPE (0041, 0125 and 0308) cells (1 x 10⁶) were transfected with pLTR-CAT (10 µg) or cotransfected with pLTR-CAT and pTAT (10 µg). Cell extracts were prepared 48 hr after transfection and analyzed for CAT activity. RD cells were included for comparison. C—chloramphenicol; AC—acetylated forms of chloramphenicol.

The low-level replication of HIV observed in some cell lines, including RPE, suggests that cell-associated factors may also play a role in supporting HIV LTR-directed expression.

Direct infection of ocular cells by HIV was also investigated. Cells exposed to the virus were monitored for the expression of viral gene products. In addition to the routine reverse transcriptase assay, we also used viral antigen assay. The HIV viral antigen assay has been reported to be more sensitive than the RT assay by several orders of magnitude. Both assays indicated lack of productive infection of cells derived from ocular tissue by HIV. We also used transactivation function assay to assess the infectibility of cells by HIV. This assay has been successfully used in our laboratory to identify cells derived from CNS tissues infectible by HIV. The transactivation function assay also showed negative results with the RPE cells, indicating the presence of tat protein below detectable levels, presence of latent viral genome or nonpermissiveness of the cells to HIV. Kennedy and colleagues analyzed retinal tissues from AIDS retinopathy patients by an in situ hybridization method. Expression of the HIV genome was not evident in retinal tissues. The sensitivity of these techniques may be a limiting factor in the identification of infected cells. Recently Pomerantz et al reported the presence of HIV-type 1 (HIV-1) in the retinas of two patients with AIDS. Our studies with a number of ocular cells showed that these cells did not show evidence of productive infection with HIV. Nonproductive infection, however, is possible. This will be looked into in the future, using polymerase chain reaction technique. It is also possible that the negative results observed may be due to the specific HIV-1 isolates used in our studies. Recently, HIV-1 isolates with restricted tropism for monocytes, glial and T4 lymphoid cells have been reported. It would be interesting to compare the biologic properties of retinal pigment epithelium cell lines

<table>
<thead>
<tr>
<th>Ocular cells used</th>
<th>Reverse transcriptase activity (cpm)</th>
<th>HIV antigen (O.D.)</th>
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<tbody>
<tr>
<td>0308</td>
<td>&lt;1000</td>
<td>0.060</td>
</tr>
<tr>
<td>0125</td>
<td>&lt;1000</td>
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</tr>
<tr>
<td>Fetal retinal cells</td>
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</tr>
<tr>
<td>Retinoblastomas cell lines</td>
<td>&lt;1000</td>
<td>0.060</td>
</tr>
<tr>
<td>Y79</td>
<td>&lt;1000</td>
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</tr>
<tr>
<td>WER1</td>
<td>&lt;1000</td>
<td>0.055</td>
</tr>
<tr>
<td>Choroidal endothelial cells</td>
<td>&lt;1000</td>
<td>0.070</td>
</tr>
<tr>
<td>Chor 55 (mix cells primary)</td>
<td>&lt;1000</td>
<td>0.065</td>
</tr>
<tr>
<td>Corneal fibroblast cells</td>
<td>&lt;1000</td>
<td>0.065</td>
</tr>
<tr>
<td>K61 (primary)</td>
<td>&lt;1000</td>
<td>0.065</td>
</tr>
</tbody>
</table>

The background of the uninfected cells, with respect to reverse transcriptase and antigen assays were <1000 cpm and <0.070 O.D. units, respectively.
HIV-1 isolates derived from specific ocular tissues and peripheral blood lymphocytes of AIDS patients.

Key words: AIDS, ocular tissues, human immunodeficiency virus (HIV), tissue culture, pigment epithelium, retina

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