HTLV-1 Infection of Human Retinal Pigment Epithelial Cells and Inhibition of Viral Infection by an Antibody to ICAM-1

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PURPOSE. To examine whether human T-cell leukemia virus type 1 (HTLV-1) could infect a human retinal pigment epithelial (RPE) cell line, ARPE-19, in vitro and to investigate its regulation.

METHODS. A coculture system with ARPE-19 and irradiated cells of an HTLV-1-producing T-cell line, MT2 was used to determine the permissivity of RPE to HTLV-1 infection in vitro. The susceptibility to HTLV-1 was assessed by detection of viral DNA using the polymerase chain reaction (PCR), viral mRNA transcripts with reverse transcription PCR (RT-PCR) and viral antigen by immunofluorescence staining. An HTLV-1 Tax-activated luciferase reporter assay was developed to measure viral infection quantitatively. The ICAM-1 expression on cocultured ARPE-19 cells was detected by flow cytometry and an ICAM-1-neutralizing antibody was used to test ICAM-1's role in the HTLV-1 infection of ARPE-19 cells. The regulation of HTLV-1 infection was investigated by culturing ARPE-19 cells with proinflammatory cytokines.

RESULTS. HTLV-1 infected ARPE-19 cells in vitro. The infection correlated with elevated expression of intercellular adhesion molecule (ICAM) on the surface of ARPE-19 cells. ICAM-1-neutralizing antibody dramatically inhibited viral infection. Furthermore, proinflammatory cytokines dramatically suppressed HTLV-1 viral infection.

CONCLUSIONS. The tropism of HTLV-1 to retinal pigment epithelium could provide an explanation for the pathogenesis of HTLV-1-related ophthalmic diseases. A better understanding of specific roles of proinflammatory cytokines in the development of ophthalmic diseases may be beneficial for treatment. (Invest Ophthalmol Vis Sci. 2006;47:1510–1515) DOI: 10.1167/iovs.05-1277

Human T-lymphotropic virus type (HTLV)-1 is associated with a variety of human diseases, including adult T-cell leukemia/lymphoma (ATL),1 the neurologic disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP),2,3 infective dermatitis,4–6 and HTLV-1-associated uveitis (HU),7–9 which is defined as uveitis of undetermined etiology in HTLV-1 carriers. The spectrum of HTLV-1 associated ophthalmic manifestations is expanding. In addition to HU, reported ocular diseases of HTLV-1 include opportunistic chorioretinal infections,10 retinal pigmentary degeneration,11,12 leukemic and lymphomatous infiltrates,13 neuroophthalmic disorders14 and keratoconjunctivitis sicca.15 HU has been observed to be very common in HTLV-1-endemic areas such as Japan.5

The retinal pigment epithelium (RPE) is located between the retinal photoreceptor layer and the vascularized choroid. It plays an important role in maintaining the homeostasis of the outer retina.16 It regulates the transport of nutrients to the photoreceptors, the phagocytosis of old rod outer segments, and the absorption of stray light.17 Furthermore, tight junctions unite RPE into an impermeable shield that prevents blood-borne cells and molecules from entering the subretinal space from the choriocapillaris.18 Because of its strategic location, the RPE frequently encounters infectious agents and inflammatory molecules. In addition to these functions, the RPE is thought to play an important role in the immune response by expressing major histocompatibility complex (MHC) molecules, adhesion molecules (such as intercellular adhesion molecule [ICAM]-1), and FasL, and producing various cytokines. Cytokines secreted by RPE cells contribute in various ways to immune and inflammatory responses. Some RPE-derived cytokines prevent or downregulate immune responses, whereas others initiate or augment immune responses.19 ARPE-19, a human RPE cell line, has been commonly used as a cell model for the study of the blood–retina barrier and pathologic conditions in vitro. It retains most of the morphologic characteristics of primary RPE cells and expresses specific RPE markers and cell surface adhesion molecules.20

The pathogenesis of HTLV-1-associated diseases is still poorly understood. Several viral and host factors, such as viral load and immune response, are believed to play a major role in disease progression. In patients with HU, HTLV-1 proviral DNA can be detected by PCR in mononuclear cells from vitreous humor.21 The interaction between HTLV-1-infected blood cells with a different kind of host cells and immune reactions mediated by these infected cells may be pathogenic mechanisms.

In this study, we demonstrated that HTLV-1 can infect ARPE-19 in vitro. We showed further that ICAM-1, also known as CD54, is involved in HTLV-1 infection of ARPE-19 cells. The expression of ICAM-1 on ARPE-19 increased after coculturing with HTLV-1 producing T-cell line MT2.22 Blockage of ICAM-1 with a neutralizing antibody decreased viral infection. Finally, we demonstrated that proinflammatory cytokines inhibit viral infection. These results may provide novel insights into the pathogenesis of HTLV-1-related ophthalmic diseases.

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**MATERIALS AND METHODS**

**Cell Culture and HTLV-1 Infection In Vitro**

MT2, an HTLV-1 infected human T-cell line, and Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin (100 U/mL each), and 2 mM L-glutamine. ARPE-19 cells (ATCC, Manassas, VA) were routinely cultured in minimum essential medium (MEM) with the same supplements. In vitro infection of HTLV-1 was performed by a standard coculture method. Briefly, ARPE-19 cells were plated and cocultured with three times the number of irradiated (9000 rads) MT2 or Jurkat cells. After 3 days of coculturing, MT2 cells were removed, and the attached ARPE-19 cells were washed, trypsinized, split one to three, and plated on new culture dishes every 3 days.

**Polymerase Chain Reaction and Reverse Transcription–Polymerase Chain Reaction**

Genomic DNA was prepared from ARPE-19 (Wizard genomic DNA purification kit; Promega, Madison, WI). PCR was performed with a master mix kit (HotStarTaq; Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA was extracted (RNeasy mini kit; Qiagen). RT-PCR was performed with a one-step kit (Promega), according to the manufacturer's instructions. The amplification procedure for RT-PCR was as follows: 48°C for 45 minutes, 94°C for 2 minutes and by 30 cycles of the following: 94°C for 30 seconds, 57°C for 1 minute, and 72°C for 1 minute. The primers used for amplification were as follows: HTLV-1 pol, 5'-GTTTACCCATTGCCGAAGC-3' and 5'-AATGGGTAATGTGCGCTTGC-3'; HTLV-1 pX, 5'-CGGATACCCAGTCTAGGTG-3’ and 5'-AGGGGATACCGGGGATCTG-3'; human CD4, 5'-GCAGCCACTGAGGAAAGAA-3' and 5'-TTGATGCAGGGGATCTT-3'.

**Immunohistochemistry**

ARPE-19 cells were cultured on plastic chamber slides (Laboratory-Tek Permanox; Nalge Nunc International Corp., Naperville, IL) for 24 hours and then cocultured with MT2 for another 72 hours. After three washes with phosphate-buffered saline (PBS), cells were fixed by cold fixing buffer (methanol/acetic acid, 1:1) at −20°C for 20 minutes and blocked with 10% FBS in PBS for 15 minutes. The cells were then incubated with 10 µg/mL of an anti-HTLV-1 p24CA gag antibody (kindly provided by Genevieve Franchini, National Cancer Institute, Bethesda, MD) at room temperature for 1 hour followed by incubation with a Cy3-conjugated anti-mouse secondary antibody (1 µg/mL) along with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) incubation (1 µg/mL, Jackson ImmunoResearch Laboratory, West Grove, PA) at room temperature for 1 hour. Nonspecific mouse IgG was used as the control at the same concentration as the primary antibody.

**DNA Transfection and Dual Luciferase Reporter Assay**

Transfection of ARPE-19 cells was performed using Nucleofector II system (Amaxa Inc., Cologne, Germany). Program T-20 was used after optimization according to the manufacturer's instructions. ARPE-19 cells (2 × 10^5) were transfected with 5 µg of an HTLV-1 LTR-firefly luciferase construct (pHTLV-Luc) and 1.5 µg of a Tkrenilla luciferase construct (phRG-TK). Transfected ARPE-19 cells were then cultured in the presence or absence of three times the number of irradiated MT2 cells. ARPE-19 without transfection but cocultured with MT2 was used as the negative control. ARPE-19 cells transfected with 1 µg of a CMV-Tax construct along with pHTLV-Luc and phRG-TK were used as the positive control.

**Effect of Reverse Transcriptase Inhibitor and Cytokines**

ARPE-19 cells were transfected with pHTLV-Luc and phRG-TK as just described and cocultured with MT2 cells. Fifty nanomolar of 3'-deoxythymidine (AZT) was added 6 hours before coculturing. Alternatively, a cocktail of proinflammatory cytokines including TNF-α, IFN-γ, and IL-1 was added before coculturing. The concentrations of three cytokine cocktails are as follows: low-IL-1, 0.2 ng/mL; TNF-α, 0.2 ng/mL; IFN-γ, 1 ng/mL; medium-IL-1, 1 ng/mL; TNF-α, 1 ng/mL; IFN-γ, 5 ng/mL; high-IL-1, 5 ng/mL; TNF-α, 5 ng/mL; and IFN-γ, 25 ng/mL. After 72 hours of coculturing, a dual-luciferase reporter assay was performed as described earlier as an indicator of HTLV-1 infection, to determine the effect of AZT and cytokines.

**Flow Cytometry**

Approximately 5 × 10^5 ARPE-19 cells after cocultivation were washed twice with 3 mL of cold PBS with 0.5% BSA, blocked with 10% mouse serum for 10 minutes in 0.1 mL of PBS with 0.5% BSA and then stained with FITC-labeled anti-human ICAM-1 for 15 minutes. After two washes with PBS and fixing with 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA), cells were acquired by a flow cytometry (FACSCaliber; BD Biosciences, San Diego, CA) and analyzed on computer (FlowJo software; TreeStar, San Jose, CA).

**RESULTS**

**Detection of HTLV-1 Proviral DNA and Gene Expression in ARPE-19 Cells Cocultured with MT2 Cells**

ARPE-19 cells were cocultured with irradiated HTLV-1-producing MT2 cells as described in the Materials and Methods section. The irradiation dosage used for MT2 cells was 9000 rads, which is lethal for all treated cells. Indeed, after 10 days of coculture, no discernable MT2 cells were seen. Trypan blue staining confirmed that no MT2 cells were viable 10 days after irradiation. This, along with repeated washing, assured that no irradiated MT2 cells persisted in the ARPE-19 culture at the time of DNA and RNA isolation 15 days after irradiation.

To examine whether HTLV-1 infection had occurred in the infected ARPE-19 cells, proviral DNA sequences of gag-pol and pX were assessed by PCR and RT-PCR. As shown in Figure 1A, in DNA samples extracted from ARPE-19 cocultured with MT2, both gag-pol and pX sequences (180 and 159 bp) of the HTLV-1 provirus DNA could be detected by PCR amplification. DNA samples prepared from ARPE-19 cocultured with Jurkat cells and untreated MT2 cells were used as the negative and positive controls, respectively. We next examined viral mRNA expression by RT-PCR for the HTLV-1 pX mRNA. A 159-bp product indicating the transcription of the pX gene was observed in ARPE-19 cocultured with irradiated MT2 cells but not in ARPE-19 cocultured with Jurkat cells (Fig. 1B). To exclude further the possibility that viral gene amplification was due to the contamination from residual MT2 cells, we used RT-PCR to amplify MT2-specific human CD4 (hCD4). As shown in Figure 1C, 188 bp of hCD4 product was amplified from MT2 cells but not from ARPE-19 cells cocultured with MT2 cells. These results suggest that ARPE-19 cells were infected by HTLV-1 after coculture.

**Active Expression of HTLV-1 Antigen in Infected ARPE-19 Cells**

To determine whether viral antigens are produced in infected ARPE-19 cells, immunofluorescence assays for p24CA gag protein were performed by indirect immunofluorescence staining. Expression of p24CA gag was detected in the cytoplasm of ARPE-19 cells 3 days after HTLV-1 infection, whereas no p24CA gag expression was observed when a control IgG was used or in control ARPE-19 without coculturing with MT-2 (Fig. 2). These observations again demonstrate that HTLV-1 infected the ARPE-19 cells.
Induction of HTLV-1 LTR Activity in ARPE-19 after Coculturing with Irradiated MT2 Cells

HTLV-1 encodes a 40-kDa nuclear protein, Tax, that transactivates viral gene transcription from three 21-bp repeats in the U3 region of the HTLV-1 long terminal repeat (LTR). To evaluate further the HTLV-1 infection of ARPE-19 cells, we developed a sensitive and quantitative method to measure viral transmission. We cotransfected a construct containing the HTLV-1 LTR regulating the expression of the firefly luciferase reporter gene (pHTLV-Luc) and another plasmid with the renilla luciferase reporter gene (phRG-TK), which was used to normalize transfection efficiency. In this assay, reporter-transfected ARPE-19 cells infected with HTLV-1 were expected to have higher firefly luciferase activity due to transactivation by the Tax protein synthesized after viral transmission.

ARPE-19 cells transfected with both pHTLV-Luc and phRG-TK constructs were cocultured with irradiated MT2, which actively produced HTLV-1 particles. Activities of the luciferase reporters were measured and the ratios of firefly luciferase activity to renilla luciferase activity were then calculated, to reflect HTLV-1 infection of ARPE-19 cells. Reporter-transfected ARPE-19 cells, after coculture with MT2 for 3 days, showed a more than 30-fold induction of firefly luciferase activity compared with the controls: nontransfected ARPE-19 cocultured with MT2 and transfected ARPE-19 without coculturing with MT2 (Fig. 3). Cotransfection of both luciferase reporter plasmids plus a Tax-expressing plasmid served as the positive control. To validate further that HTLV-1 viral infection, reverse transcription, and integration had taken place, we treated ARPE-19 cells with 50 nM 3′/H11032-azido-3′/H11032-deoxythymidine (AZT, a thymidine analogue, acted as reverse transcriptase inhibitor to inhibit virus replication) 6 hours before coculturing with MT2. As shown in Figure 3, firefly luciferase activity in ARPE-19 cells cocultured with MT2 was greatly inhibited by
AZT. By contrast, AZT had no effect on HTLV-1 LTR transactivation by transfected Tax-expressing construct. These results confirm that ARPE-19 cells were infected by HTLV-1, and viral replication occurred in the cells after MT2 coculturing.

Involvement of ICAM-1 in HTLV-1 Viral Infection

It is well known that HTLV-1 transmission occurs primarily through cell–cell contact, whereas free HTLV-1 virions infect susceptible cells very inefficiently.28,29 Adhesion molecules, such as ICAM-1, is known to mediate cell–cell interactions, particularly those between T cells and antigen-presenting or target cells.30 We tested whether ICAM-1 was involved in HTLV-1 viral infection of ARPE-19 cells and its role in this process.

First, we examined surface expression of ICAM-1 on cocultured ARPE-19 cells by flow cytometry. ARPE-19 cells were cocultured with irradiated MT2 for 1, 2, and 3 days. As shown in Figure 4A, ARPE-19 cells constitutively expressed ICAM-1. ICAM-1 expression on ARPE-19 cells increased substantially 1 day after coculturing with MT2 cells, reached peak on the second day, and remained plateaued on the third day. This indicated that ICAM-1 was upregulated in ARPE-19 cells after coculturing. We next investigated potential functions of ICAM-1 in HTLV-1 infection of ARPE-19 cells. A mouse monoclonal anti-human ICAM-1 antibody that blocks cell adhesion was preincubated with ARPE-19 cells transfected with the luciferase-reporters, pHTLV-Luc and phRG-TK. Six hours after the addition of ICAM-1 antibody, irradiated MT2 cells were added to ARPE-19 culture. Luciferase activities were measured after 3 days of coculturing. As indicated in Figure 4B, HTLV-Luc activity was dramatically reduced by the ICAM-1-blocking antibody. In contrast, HTLV-Luc activity was not affected when transfected ARPE-19 cells were incubated with an equal amount of isotype control antibody. These data suggest that ICAM-1 acts on the early stage of transmission during HTLV-1 infection of ARPE-19 cells. Overall, these results strongly suggest that ICAM-1 plays a critical role in ARPE-19 infection by HTLV-1.

Effect of Proinflammatory Cytokines on HTLV-1 Viral Infection of ARPE-19 Cells

T-cell clones (TCCs) established from the intraocular fluid of patients with HTLV-1 uveitis have been shown to produce high amounts of IL-1α, TNF-α, and IFN-γ constitutively.31,32 We asked...
In this study, the evidence showed that HTLV-1 can infect human ARPE-19 cells. The infection of ARPE-19 cells with HTLV-1 is supported by (1) the detection of HTLV-1 proviral DNA, mRNA, and p24CA in the infected cells; (2) the ability of the infected cells to support transactivation of the luciferase reporter construct (pHTLV-Luc) wherein the firefly luciferase gene has been placed under the control of the Tax-responsive HTLV-1 LTR; and (3) the inhibition of viral infection by AZT, a reverse transcriptase inhibitor. We further demonstrate that HTLV-1-infected cells could express excessive amounts of ICAM-1 and LFA-1, suggesting a potential cellular protective mechanism to inhibit further spread of viral infection. It has been reported, however, that a human ocular tissue could be infected by HTLV-1 with active viral reverse transcription and integration in infected ocular tissues. The infection was demonstrated by the fact that viral transcript was detected, new viral protein was synthesized in infected cells, and the reverse transcriptase inhibitor AZT inhibited HTLV-1 viral infection of ARPE-19 cells. This finding suggests the possibility that RPE can be infected by HTLV-1 after coming into contact with HTLV-1-infected CD4+ T-cells and may be the potential reservoir for HTLV-1. In this line, it is not difficult to propose that HTLV-1 infection in RPE cells may contribute to the chronic activation and destruction of eye tissues by a cellular immune response against HTLV-1, which was already observed in HTLV-1-related ocular diseases.35 HTLV-1 specific CD8⁺ cytotoxic T lymphocytes (CTLs) are likely to play an important role in this scenario.36 Further, HTLV-1-infected cells and activated T-cells may also secrete cytokines to cause tissue inflammation and damages. Our results showed that the proinflammatory cytokine cocktail (IL-1, IFN-γ, and TNF-α) greatly suppressed HTLV-1 viral infection, suggesting a potential cellular protective mechanism to inhibit further spread of viral infection. It has been reported, however, that HTLV-1-infected cells could express excessive amounts of cytokines such as IL-1, IL-6, IFN-γ, TNF-α, among others.37 These cytokines have been implicated in the development and pathogenesis of various forms of HTLV-1-related uveitis. Therefore, proinflammatory cytokines secreted by host cells after HTLV-1 infection may play dual roles in the pathogenesis of HTLV-1-associated ocular diseases.

Taken together, the results in this study suggest that ocular tissues could be a potential host for HTLV-1. The regulation of HTLV-1 infection demonstrated herein may help us to gain a better understanding of the pathogenic mechanisms of HTLV-1-related ocular diseases.

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