Proinflammatory Cytokine Gene Induction by Human T-Cell Leukemia Virus Type 1 (HTLV-1) and HTLV-2 Tax in Primary Human Glial Cells[▽]

Prabal Banerjee,¹ Rosemary Rochford,¹ J. Antel,³ G. Canute,² Stephen Wrzesinski,¹ Michelle Sieburg,¹ and Gerold Feuer^{1*}

Department of Microbiology and Immunology¹ and Department of Neurosurgery,² SUNY Upstate Medical University, Syracuse, New York 13210, and Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada³

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Infection with human T-cell leukemia virus type 1 (HTLV-1) can result in the development of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic inflammatory disease of the central nervous system (CNS). HTLV-2 is highly related to HTLV-1 at the genetic level and shares a high degree of sequence homology, but infection with HTLV-2 is relatively nonpathogenic compared to HTLV-1. Although the pathogenesis of HAM/TSP remains to be fully elucidated, previous evidence suggests that elevated levels of the proinflammatory cytokines in the CNS are associated with neuropathogenesis. We demonstrate that HTLV-1 infection in astroglomas results in a robust induction of interleukin-1 β (IL-1 β), IL-1 α , tumor necrosis factor alpha (TNF- α), TNF- β , and IL-6 expression. HTLV encodes for a viral transcriptional transactivator protein named Tax that also induces the transcription of cellular genes. To investigate and compare the effects of Tax1 and Tax2 expression on the dysregulation of proinflammatory cytokines, lentivirus vectors were used to transduce primary human astrocytomas and oligodendrogliomas. The expression of Tax1 in primary human astrocytomas and oligodendrogliomas resulted in significantly higher levels of proinflammatory cytokine gene expression compared to Tax2. Notably, Tax1 expression uniquely sensitized primary human astrocytomas to apoptosis. A Tax2/Tax1 chimera encoding the C-terminal 53 amino acids of the Tax1 fused to the Tax2 gene (Tax²²¹) demonstrated a phenotype that resembled Tax1, with respect to proinflammatory cytokine gene expression and sensitization to apoptosis. The patterns of differential cytokine induction and sensitization to apoptosis displayed by Tax1 and Tax2 may reflect differences relating to the heightened neuropathogenicity associated with HTLV-1 infection and the development of HAM/TSP.

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of a slow progressive neurological disease, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (42). Although most HTLV-1-infected individuals remain asymptomatic carriers, an estimated 5 to 10% of HTLV-1-infected individuals develop HAM/TSP. HTLV-2 was originally isolated from a patient with an atypical hairy cell leukemia and shares ca. 70% sequence homology with HTLV-1. To date, HTLV-2 infection has been rarely and controversially linked to the development of neurodegenerative diseases (2, 30).

Cytokines play a critical role in physiological processes in the central nervous system (CNS), including establishing and maintaining normal homeostasis, as well as mediating inflammatory and immune responses. Cytokines have also been implicated as being the major mediators of demyelination in the CNS, resulting in a variety of inflammatory and neoplastic diseases (1). Elevated levels of tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), IL-1 α , and IL-6 have previously been detected in the cerebrospinal fluid of HAM/TSP

patients (28, 40, 57), suggesting that increased expression of these proinflammatory cytokines may be involved in the development of HAM/TSP. TNF- α has been shown to adversely affect glutamate metabolism in astrocytes that were placed in transient contact with HTLV-1-infected T cells and has also been shown to enhance the apoptosis of oligodendrocytes in the spinal cord of HTLV-1-infected WKAH rats (21, 54). TNF- α also enhances the transmigration of HTLV-1-infected T lymphocytes into the CNS through the blood-brain barrier (BBB) (49). Similarly, expression of IL-1 has been associated with the induction of adhesion molecules and the destruction of the integrity of the BBB, resulting in increased migration of lymphocytes into the CNS (26, 46). IL-6 is a regulator of inflammation and immune response, and its abnormal expression is linked to the loss of BBB integrity and spinal cord injury in a variety of CNS autoimmune diseases (20, 24).

The HTLV-1 Tax oncoprotein (Tax1) is a transactivator of viral transcription and also induces the expression of a variety of cellular genes by activation of the NF- κ B and CREB/ATF pathways. The HTLV-2 Tax protein (Tax2) shares ca. 78% homology with Tax1, and both proteins demonstrate remarkable similarities in their patterns of transcriptional transactivation (33). Tax1 demonstrates a more robust phenotype with respect to the inhibition of p53, the suppression of multilineage hematopoiesis in vitro, the transformation of rat fibroblasts, micronucleus formation, and the induction of cell cycle arrest

* Corresponding author. Mailing address: Department of Microbiology and Immunology, SUNY Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210. Phone: (315) 464-7671. Fax: (315) 464-7682. E-mail: feuer@upstate.edu.

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in human CD34⁺ hematopoietic progenitor cells in comparison to Tax2 (11, 34, 50, 60, 61). Tax1 and Tax2 also demonstrate unique patterns of subcellular localization in lymphoid cells (35).

Previous studies have shown that human astroglomas can be infected with HTLV-1 *in vitro* (55, 63) and that infection correlates with the induction of the proinflammatory cytokines TNF- α and IL-1 α (36, 53). To systematically evaluate the role of Tax1 and Tax2 expression in neuroglial cells, a lentiviral vector system capable of cotransducing the HTLV-1 Tax and the green fluorescent protein (GFP) reporter gene was used. Tax1 transduction resulted in a robust induction of proinflammatory cytokines in human astrocytes and oligodendrocytes, in contrast to Tax2. Tax1 also uniquely sensitized primary human astrocytomas to serum withdrawal-mediated apoptosis. We postulate that the elevated activity of Tax1 in mediating proinflammatory cytokine gene expression and sensitization to apoptosis contributes to the development of HAM/TSP.

MATERIALS AND METHODS

Cell lines and primary cells. Human U251 astrogloma cells and MO3.13 oligodendroglial cell line were cultured in high glucose (4,500 mg/liter) Dulbecco modified Eagle medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS; Gemini, Calabasas, CA), 2 mM L-glutamine (Gibco-BRL), penicillin (100 U/ml; Gemini), and streptomycin (100 μ g/ml; Gemini) at 37°C in a humidified incubator with 5% CO₂. Primary human astrocytomas were prepared from biopsies of fresh human tumor samples (10). Single-cell suspensions were prepared from these tumors as previously described (9). Astrocytomas stained positive for glial fibrillary acidic protein (GFAP). Primary human oligodendroglomas were from patients with type IV anaplastic oligodendroglioma (WHO grade IV), obtained in accordance with Upstate Medical University IRB approved guidelines. Oligodendroglomas stained positive for vimentin and negative for GFAP, confirming their oligodendrocytic lineage. Cells were cultured in high glucose (4,500 mg/liter) DMEM (Gibco-BRL) supplemented with 10% heat-inactivated (56°C, 30 min) FBS (Gemini), 2 mM L-glutamine (Gibco-BRL), penicillin (100 U/ml; Gemini), streptomycin (100 μ g/ml; Gemini), 25 mM HEPES buffer, and nonessential amino acids (10 mM; Gibco-BRL). Human fetal astrocytes (12- to 14-week gestations) were obtained according to Medical Research Council of Canada-approved guidelines. Central nervous tissue was stripped of meninges and blood vessels, mechanically dissociated with scalpel blades, and then treated with trypsin (0.25%) and DNase (50 μ g/ml) at 37°C for 45 min. Dissociated tissue was passed through a 130- μ m-pore-size mesh and washed twice in phosphate-buffered saline (PBS), and cells initially consisting of astroglia, neurons, and sparse microglia were plated directly onto poly-L-lysine-coated tissue culture flasks in DMEM without FBS. Cultures were allowed to become confluent and then dispersed by using 0.25% trypsin. Astrocytes (95% pure, as determined by GFAP immunoreactivity) were treated with trypsin and reseeded onto poly-L-lysine-coated 16-well chamber glass slides at 10⁵ cells per well.

HTLV infection of U251 astrogloma cells. U251 astroglomas were cocultured with lethally irradiated (10³ rads) SLB-1 (HTLV-1-infected) and 729/pH6Neo (HTLV-2-infected) cells at a recipient/donor ratio of 1:5, as previously described (29, 60). After 1 week, the cultures were washed with PBS, and the medium was replaced. Freshly irradiated SLB-1 or 729/pH6Neo cells were added again to the U251 astroglomas at a recipient/donor ratio of 1:5. After one more week, the culture was washed with PBS, and the medium was replaced. To confirm the lack of viability of lethally irradiated HTLV-1 and -2-infected donor cells, normal and irradiated SLB-1 and 729/pH6Neo cells (without U251 astroglomas) were analyzed for viability by staining with phycoerythrin (PE)-conjugated annexin V (Biovision, Mountain View, CA) and 7-amino actinomycin D (7-AAD; Calbiochem, La Jolla, CA) 1 week postirradiation as described previously (51). After 14 days U251 astroglomas cultured with or without the donor cells were washed twice with PBS and were permeabilized or fixed by using a Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA) in accordance with the manufacturer's protocol. Briefly, cells (10⁶) were pelleted by centrifugation (250 $\times$ g), washed twice with PBS, and resuspended in 250 μ l of Cytofix/Cytoperm (BD Biosciences) solution for 20 min at 4°C. Cells were then washed twice with permeabilization-wash buffer (Cytofix/Cytoperm kit; BD Biosciences) and resuspended

in 100 μ l of permeabilization/wash buffer. Cells (10⁶) were then incubated with either mouse anti-HTLV-1 p19^{gag} monoclonal antibody (MAb) or mouse anti-HTLV-2 p19^{gag} MAb (Zeptomatrix, Buffalo, NY) at 3 μ l/sample for 30 min at 4°C, washed twice with PBS, and then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G MAb (1 μ l/sample; Dako A/S, Glostrup, Denmark) for 30 min in the dark at 4°C (60). Cells were pelleted by centrifugation (250 $\times$ g) in a Beckman GPR centrifuge and resuspended in fluorescence-activated cell sorting buffer for flow cytometric analysis.

Apoptosis analysis of primary human astrocytomas. Human astrocytomas were infected with lentivirus vector virus (LV) stocks (MOI = 3) by spinfection in a Beckman GPR centrifuge (840 $\times$ g) in a final volume of 2.0 ml of serum-free DMEM for 2 h at room temperature (51). Cells were then washed twice with DMEM with 10% FBS and cultured for 24 h in the same media. For apoptosis studies the cells were then resuspended in DMEM without 10% FBS for 24 h. Apoptosis analysis on human astrocytomas was performed as described above and previously (51). Cells were analyzed on a LSR II flow cytometer (Becton Dickinson, San Jose, CA).

Generation of VSV-G-pseudotyped lentiviral vectors and infection of primary astrocytomas and oligodendroglomas. Vesicular stomatitis protein G (VSV-G)-pseudotyped LV stocks were generated as previously described (6, 51, 61). Briefly, a three-plasmid transfection system was used consisting of the transfer plasmid [pHR' cytomegalovirus (CMV)-GFP, pHR' CMV-Tax1/GFP, pHR' CMV-Tax1(-)/GFP, pHR' CMV-Tax2/GFP, or pHR' CMV-Tax221/GFP], a packaging vector (pCMV Δ R8.2 Δ VPR), and a vector encoding the VSV-G envelope protein (pHCMV-G) (7). Plasmids were cotransfected into 293T (10⁷) cells by using Lipofectamine 2000 (Invitrogen/Life Technologies, Carlsbad, CA) (60). Supernatants were harvested at 2 and 4 days posttransfection and filtered through a 0.45- μ m-pore-size filter, pooled, and subjected to ultracentrifugation (50,000 $\times$ g for 4 h) in a SW27 rotor (Beckman, Palo Alto, CA). The pellet was resuspended in 1/100 initial volume in serum-free DMEM overnight at 4°C and pooled and frozen at -80°C. Titers of virus stocks were determined by infecting HeLa cells (3 $\times$ 10⁵) with virus stocks that were serially diluted (1:10, 1:100, 1:500, and 1:1,000) in DMEM. HeLa cells were analyzed for GFP expression at 48 h postinfection by flow cytometry. Virus titers ranged between 10⁶ and 10⁷ transducing units per ml. Primary astrocytomas and oligodendroglomas were infected with lentivirus vectors (multiplicity of infection [MOI] = 3) by spinfection in a Beckman GPR centrifuge (840 $\times$ g) in a final volume of 2.0 ml of serum-free DMEM for 2 h at room temperature. Cells were then washed twice with DMEM with 10% FBS and cultured for 72 h in the same media.

Transient-transfection assays and RPA. U251 astrogloma and MO3.13 oligodendroglial cells were transfected with LV constructs by using Lipofectamine 2000 (Invitrogen/Life Technologies), as described previously (60). Briefly, cells (10⁵) were plated in 2 ml of DMEM with 10% FBS, 2 mM L-glutamine, and 100 μ g of penicillin-streptomycin/ml in a six-well culture plate and transfected with 4 μ g of LV vector constructs. Total RNA (10 μ g) was extracted from the cells 72 h posttransfection or postinfection (in case of primary cells) by using TRIzol (Gibco-BRL) according to the manufacturer's instructions. Cytokine levels were assayed by using a cytokine-specific RNase protection assay (RPA) as described previously (48). Briefly, a cytokine-specific riboprobe template set (HL-14) was assembled from EcoRI-linearized and purified subclones. The HL-14 template set was used to synthesize riboprobes specific for IL-6, IL-1 α , IL-1 β , TNF- β , TNF- α , transforming growth factor β (TGF- β), and L-32. All of the riboprobe syntheses were driven by T7 bacteriophage RNA polymerase with [α -³²P]UTP as the labeling nucleotide (19). The subsequent steps of probe purification, RNA probe hybridization, RNase treatment, purification of protected RNA duplexes, and resolution of protected probes by denaturing polyacrylamide gel electrophoresis were performed as described previously (19). Protected probe bands were visualized by autoradiography (Blue sensitive film; LPS, Rochester, NY) and were quantified by using a PhosphorImager 445 Si (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics). The ImageQuant software was used to measure volume of rectangular objects to generate PhosphorImager counts used to calculate the signal present in each lane and standardize the values to an internal housekeeping gene signal (L-32). The signal was standardized to mock samples, and the fold induction was calculated with respect to Tax1(-) (antisense vector)-transfected or -transduced cells as a negative control.

ELISA. U251 cells were transfected and primary astrocytomas or fetal astrocytes were infected with lentiviral vectors as described above. Culture fluids were harvested 24, 48, and 72 posttransfection or postinfection (in the case of primary cells) and assayed by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The fold increase in the level of secreted cytokines (in pg/ml) was calculated with respect to Tax1(-) (antisense vector)-transfected or -transduced cells.

FIG. 1. HTLV-1 and -2 infections of U251 astrogloma cells induce proinflammatory cytokine gene expression. U251 astrogloma cells were infected with HTLV-1/-2 by cocultivation with lethally irradiated (10^3 rads) HTLV-1-infected (SLB-1) and HTLV-2-infected (729/pH6Neo) donor cells. After 2 weeks of cocultivation, cells (10^6) were stained with a primary mouse anti-human HTLV-1 or HTLV-2 p19^{gag} antibody (ZeptoMatrix Corp., Buffalo, NY), followed by secondary fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (Dako) and analyzed by flow cytometry. (A and B) HTLV p19^{gag} expression on HTLV-1-infected (A) and HTLV-2-infected (B) astroglomas. The orange line represents mock-infected cells, and the green line represent cells stained with secondary antibody alone in the absence of primary antibody. (C) Quantification of the expression of proinflammatory cytokines by RPA. RNA was extracted from infected astrogloma cells 2 weeks postinfection using the TRIZOL reagent (Gibco-BRL) and subjected to RNase protection using 32 P-labeled HL-14 probes as described in Materials and Methods. mRNA species were run on a 5% acrylamide urea gel, and probe bands were quantified by using a PhosphorImager 445SI and the ImageQuant 5.1 program (both from Molecular Dynamics). The experiments were performed four times, and the error bars represent the standard error of the mean (SEM). Statistical analysis was performed by using single-tail analysis of variance (ANOVA) with mock-infected cells as a negative control (*, $P < 0.05$), followed by the post-hoc Tukey test (honestly significant difference) using HTLV-2-infected cells as a negative control (**, $\alpha = 0.05$).

RESULTS

Induction of proinflammatory cytokine gene expression in HTLV-1- and HTLV-2-infected astroglomas. To compare and contrast the role of HTLV-1 and HTLV-2 infection on cytokine dysregulation in astrocytes, U251 astrogloma cells were infected with HTLV-1 or HTLV-2 by cocultivation with lethally irradiated SLB-1 (HTLV-1-infected cell line) or 729/pH6Neo (HTLV-2-infected cell line) donor cells (29, 60). Target U251 cells showed HTLV p19^{gag} expression after cocultivation with SLB-1 and 729/pH6Neo donor cells (92 and 94%, respectively), suggesting similar levels of virus

transmission and infection (Fig. 1A and B). RNA was extracted from U251 cells cultured with or without the donor cells and analyzed by RNase protection assay (RPA) 2 weeks postinfection. HTLV-1-infected U251 cells demonstrated significantly higher levels of IL-1 β , IL-1 α , TNF- α , TNF- β , and IL-6 expression compared to mock-infected cells (7-, 10-, 3.2-, 11-, and 5.2-fold induction, respectively) (Fig. 1C). In comparison, HTLV-2-infected U251 demonstrated a similar but more modest induction profile of proinflammatory cytokine gene expression (Fig. 1C). Both HTLV-1 and HTLV-2 caused a relatively small induction of TGF- β in U251 cells (1.9- and 1.8-fold, respectively). These re-

FIG. 2. Schematic representation of lentiviral vectors. Bicistronic LVs encode an internal ribosomal entry site (IRES) sequence from the encephalomyocarditis virus. Transcription of both the target and the reporter GFP gene is initiated by an immediate-early cytomegalovirus promoter (IE CMV promoter). The construction and characterization of pHR'CMV-GFP (GFP), pHR'CMV-Tax1-GFP (Tax1), pHR'CMV-Tax1(-)-GFP [Tax1(-)], and pHR'CMV-Tax2-GFP (Tax2) have been described previously (61). pHR'CMV-Tax²²¹-GFP (Tax²²¹) encodes a Tax2/Tax1 chimera consisting of the Tax2B (1 to 300 amino acids) cDNA fused in frame to the last 53 terminal amino acids of Tax1, as previously described (11). SA, splice donor; SD, splice donor; RRE, HIV-1 Rev response elements; ψ , RNA packaging site.

sults suggest that HTLV-1 induces a more robust pattern of proinflammatory cytokine expression after the infection of human astrogloma cells.

Tax transduction of human astrocytes. The HTLV-1 viral oncoprotein (Tax1) has been shown to transactivate the transcription of various cellular genes, including those that regulate cell growth and proliferation (32). Although the HTLV-2 viral oncoprotein (Tax2) shares 78% amino acid homology with Tax1, distinct phenotypic differences in the activities of these viral oncoproteins have been described (11, 34, 39, 50, 51, 60). To directly assess and compare the activities of Tax1 and Tax2 on proinflammatory cytokine gene expression, LVs encoding Tax1 or Tax2 were utilized (Fig. 2) (61). These LVs have previously been shown to express similar levels of functional Tax protein (61). U251 human astrogloma cells were transfected with LV constructs encoding GFP, Tax1, Tax2, or Tax1(-) (an antisense Tax1 construct). The transfection efficiency was $70\% \pm 5\%$ for all LV constructs, as determined by GFP expression 24 h posttransfection (data not shown). Transfection of Tax1 into U251 cells resulted in significantly higher levels of IL-1 β (3.8-fold), IL-1 α (3.0-fold), TNF- α (3.3-fold), TNF- β (2.6-fold), and IL-6 (2.9-fold) in comparison to Tax1(-)-transfected cells (Fig. 3A). In contrast, Tax2 transduction resulted in the induction of IL-1 β (2.2-fold), IL-1 α (1.8-fold), TNF- α (1.9-fold), TNF- β (1.5-fold), and IL-6 (1.6-fold) compared to Tax1(-)-transduced cells (Fig. 3A), which were at more modest levels compared to Tax1. The levels of TNF- α and IL-6 mRNA induction in Tax1- and Tax2-transfected U251 cells were confirmed by real-time reverse transcription-PCR (data not shown). Notably, the level of TGF- β expression was not significantly altered in Tax1- or Tax2-trans-

fected cells. These results demonstrate that although both Tax1 and Tax2 induce a similar pattern of proinflammatory cytokine gene expression, Tax1 induces a significantly more robust expression of all proinflammatory cytokines assayed in astrocytic cell lines in comparison to Tax2.

To determine whether Tax transduction in primary human astrocytes mimics the dysregulation of proinflammatory cytokines seen in astrocytic cell lines, astrocytomas derived from human brain tumor biopsies were infected with LVs (MOI = 3). A total of $50\% \pm 5\%$ of astrocytomas were transduced with LVs 2 days postinfection, as determined by quantification of GFP expression using fluorescence microscopy (data not shown). Tax1 expression in primary human astrocytomas resulted in significantly higher levels of IL-1 β (2.6-fold), IL-1 α (4-fold), TNF- α (2.9-fold), TNF- β (3.5-fold), and IL-6 (4.1-fold) compared to Tax1(-)-expressing cells (Fig. 3B). The levels of cytokine induction demonstrated by Tax2 were significantly higher than Tax1(-) but substantially lower than Tax1-transduced cells (Fig. 3B). TGF- β induction was not significantly altered after transduction of primary astrocytomas.

To verify that mRNA induction results in the increased secretion of proinflammatory cytokines, U251 astrogloma cells were transfected with lentiviral vectors and primary astrocytomas and fetal astrocytes from brain explant cultures were infected with LVs encoding GFP, Tax1, Tax2, and Tax1(-) (MOI = 3). At 24, 48, and 72 h posttransfection or postinfection, supernatants were collected and evaluated by ELISA. Levels of secreted IL-1 α were increased 2.3-, 2.5-, and 2.8-fold at 24, 48, and 72 h posttransfection, respectively, from Tax1-transfected U251 astrogloma cells compared to Tax1(-)-transfected cells (Fig. 4A). The level of induction of

FIG. 3. Tax-mediated induction of proinflammatory cytokine gene expression in astrocytes. (A) Astrogloma cells (U251) were transfected with LVs encoding GFP, Tax1, Tax2, Tax1(-), and Tax²²¹, using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. The cells were assessed for GFP expression at 24 h posttransfection. RNA was extracted 72 h posttransfection, and cytokine gene expression was assayed by RPA. The experiments were performed three times, and error bars represent the SEM. (B) Primary human astrocytomas were infected with LVs encoding GFP, Tax1, Tax2, and Tax1(-) by spinfection for 2 h (MOI = 3). RNA was extracted from these cells 72 h postinfection using TRIZOL (Gibco), and cytokine gene expression was analyzed by RPA. The experiments were performed three times, and error bars represent the SEM. Statistical analysis was performed by using single-tail ANOVA using Tax1(-)-transduced cells as a negative control (*, $P < 0.05$), followed by the post-hoc Tukey test (honestly significant difference) using Tax2-transduced cells as a negative control (**, $\alpha = 0.05$).

IL-1 α was significantly more modest in Tax2-transfected cells (1.4-, 1.7-, and 1.8-fold, respectively). Similarly, IL-1 α mRNA induction correlates to the increased secretion levels of IL-1 α in Tax-transduced primary human astrocytomas. IL-1 α protein levels were increased by 1.7-, 3.3-, and 3.5-fold at 24, 48, and 72 h postinfection, respectively, in Tax1-transduced primary astrocytomas and by 1.2-, 2.0-, and 2.8-fold (24, 48, and 72 h postinfection, respectively) in Tax2-transduced primary astrocytomas (Fig. 4B). Secreted levels of TNF- α and IL-6 were elevated by 3.6- and 1.5-fold, respectively, in Tax1-transduced

fetal astrocytes in comparison to Tax1(-)-transduced cells (Fig. 4C). Thus, secretion of proinflammatory cytokines is significantly higher in Tax1-expressing cells and reflects the mRNA induction levels.

The Tax 1 C terminus mediates the transcriptional trans-activation of proinflammatory cytokine gene expression. The HTLV Tax2 (Tax2A) protein is 22 amino acids shorter than Tax1. The C terminus of Tax1 encodes for a transcription factor domain (PCAF) and for a PDZ-binding motif, both of which are absent in Tax2 (15). This unique region of Tax1 has

FIG. 4. Tax-mediated secretion of proinflammatory cytokines in astrocytes. (A) Levels of secreted IL-1 α in Tax-transfected astroglomas. Astrogloma cells (U251) were transfected with LVs encoding Tax1, Tax2, and Tax1(-) using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. The cells were assessed for GFP expression at 24 h posttransfection, and the supernatant was collected at 24, 48, and 72 h posttransfection and assayed by ELISA (R&D Systems). (B) Levels of secreted IL-1 α in Tax-transduced primary astrocytomas. Primary human astrocytomas were infected with LVs encoding Tax1(-), Tax1, and Tax2 by spinfection for 2 h (MOI = 3), and the supernatant was collected 24, 48, and 72 h postinfection and evaluated for secreted IL-1 α levels by ELISA (R&D Systems). (C) Primary human fetal astrocytes were infected with LVs encoding Tax1(-) and Tax1 for 2 h (MOI = 3), and the supernatant was collected at 24, 48, and 72 h postinfection and evaluated for secreted TNF- α and IL-6 levels by ELISA (Pharmingen). Transfection and infections were performed in triplicate, and ELISA was performed on each of the samples in duplicate. The error bar represents the standard deviation. Statistical analysis was performed by using single-tail ANOVA using Tax1(-)-transduced cells as a negative control (*, $P < 0.05$), followed by the post-hoc Tukey test (honestly significant difference) using Tax2-transduced cells as a negative control (**, $\alpha = 0.05$).

been implicated in elevating the transformation efficiency in rat fibroblasts, in the suppression of hematopoiesis, and in the transcriptional transactivation of p21^{cip1/waf1} and p27^{kip1} cdk inhibitor genes (11, 60). To determine whether the C terminus of Tax1 modulates the expression of proinflammatory cytokines, U251 cells were transfected with Tax²²¹, a Tax1/Tax2 chimera that encodes the first 300 amino acids of the Tax2B gene fused in frame with the last 53 C-terminal amino acids of Tax1 (Fig. 2) (11). Tax²²¹-transfected U251 cells displayed significantly higher levels of proinflammatory cytokine expression than did Tax1(-)-transduced cells (IL-1 β [3.2-fold], IL-1 α [2.5-fold], TNF- α [2.6-fold], TNF- β [1.9-fold], and IL-6 [2.3-fold]) (Fig. 3A). The level of gene expression induced by Tax²²¹ was similar to the induction profile displayed by Tax1 and was significantly higher than the levels induced by Tax2. This suggests that domains localized within the C terminus of HTLV-1 Tax play a functional role in the transcriptional transactivation of proinflammatory cytokine gene expression.

Tax1 sensitizes primary astrocytomas to apoptosis. Tax1 has previously been shown to protect lymphoid cells from

apoptosis (51, 59). To characterize the role of Tax1 and Tax2 in modulating stress-mediated apoptosis in neuroglial cells, astrocytomas derived from human brain tumor biopsies were infected with LVs (MOI = 3) and cultured in serum-free media for 24 h. Tax1-transduced astrocytes displayed significantly higher levels of apoptosis after serum withdrawal, in contrast to Tax2- or Tax1(-)-transduced astrocytes (Fig. 5). Interestingly, Tax²²¹-transduced astrocytomas were also sensitized to apoptosis, suggesting that the C terminus of HTLV-1 Tax has a role in conferring this phenotype.

Tax expression induces proinflammatory cytokine gene expression in oligodendrocytes. Oligodendrocytes play a pivotal role in myelin generation in the CNS, and their destruction may result in the demyelination observed in HAM/TSP patients. To establish whether Tax could modulate the expression of proinflammatory cytokines in oligodendrocytes, MO3.13 human oligodendroglial cells were transfected with LVs encoding GFP, Tax1, Tax2, Tax²²¹, or Tax1(-). The transfection efficiency was 65% ($\pm 5\%$) for the LV constructs as determined by GFP expression 24 h posttransfection (data not shown). Tax1

FIG. 5. Apoptosis analysis of Tax-transduced primary human astrocytomas. Human astrocytomas (10^6) were infected with LVs encoding Tax1, Tax2, and Tax²²¹ or with the antisense vector, Tax1(-) (MOI = 3), in 2 ml of serum-free DMEM for 2 h. Cells were suspended in DMEM with 10% FBS for 24 h and then resuspended in DMEM without 10% FBS for 24 h. Cells were then stained with PE-conjugated annexin V and 7-AAD and analyzed by flow cytometry. (A) Dot plot of representative PE-conjugated annexin V versus 7-AAD flow cytometric analysis of 10^4 GFP-positive gated cells at 24 h after serum withdrawal. "Normal" refers to uninfected human astrocytomas grown in DMEM with 10% FBS for 48 h. The numbers represent the percentage of cells in each quadrant. (B) Quantification of apoptotic cells. Experiments were performed three times, and error bars represent the SEM. Statistical analysis was performed by using single-tail ANOVA with Tax1(-)-transduced cells as a negative control (*, $P < 0.05$), followed by the post-hoc Tukey test (honestly significant difference) using Tax-2-transduced cells as a negative control (**, $\alpha = 0.05$).

transfection in MO3.13 cells resulted in significant induction of TNF- α (3.6-fold), TNF- β (2.9-fold), and IL-6 (2.8-fold) (Fig. 6A). Transfection of Tax2 presented a similar but less robust pattern of proinflammatory cytokine gene expression. The transfection of Tax²²¹ resulted in the induction of TNF- α (2.4-fold), TNF- β (2.1-fold), and IL-6 (2.3-fold) at levels that were reflective of the Tax1 profile (Fig. 6A). Interestingly, no significant induction of IL-1 α or IL-1 β was detected in these cells.

To evaluate whether Tax could modulate the expression of proinflammatory cytokines in primary oligodendrocytes, human brain tumor-derived oligodendrogliomas were infected with LVs (MOI = 3; transduction efficiency of $55\% \pm 5\%$). Tax1 expression in primary oligodendrogliomas resulted in the induction of IL-1 β (3.7-fold), TNF- α (3.3-fold), TNF- β (1.7-fold), and IL-6 (2.5-fold) (Fig. 5B). Tax2 transduction resulted

in only a modest induction of IL-1 β (2.1-fold), TNF- α (1.6-fold), TNF- β (1.2-fold), and IL-6 (1.4-fold) in primary oligodendrogliomas (Fig. 6B). Interestingly, both Tax1 and Tax2 induced IL-1 β expression in primary oligodendrogliomas, indicating a differential pattern of cytokine dysregulation between primary oligodendrogliomas and oligodendrocytic cell lines. These results demonstrate that Tax expression in human oligodendrocytes results in a discernible pattern of induction of proinflammatory cytokine gene expression.

DISCUSSION

A robust induction of proinflammatory cytokines and increased sensitization to apoptosis as a result of HTLV-1 infection in neuroglial cells may reflect the heightened neuropatho-

FIG. 6. Proinflammatory cytokine gene expression in Tax-transduced oligodendrocytes. (A) Oligodendroglial cells (MO3.13) were transfected with lentiviral vectors encoding Tax1, Tax2, Tax1(-), and Tax²²¹. RNA was extracted at 72 h posttransfection using TRIZOL (Gibco-BRL), and proinflammatory cytokine gene expression was analyzed by RPA. (B) Primary oligodendroglia were infected with LVs (MOI = 3) for 2 h, and RNA was extracted at 72 h postinfection and analyzed by RPA. The experiments were performed three times, and the error bars represent the SEM. Statistical analysis was performed by using single-tail ANOVA with Tax1(-)-transduced cells as a negative control (*, $P < 0.05$), followed by the post-hoc Tukey test (honestly significant difference) using Tax2-transduced cells as a negative control (**, $\alpha = 0.05$).

genicity associated with HTLV-1 infection in contrast to infection with HTLV-2. Although inflammation triggered by the host immune response to viral infection is suggested to be involved in the pathogenesis of HAM/TSP, the early events associated with infection leading to viral entry into the CNS are largely uncharacterized (56). The ability of HTLV-1 to enter the CNS is a critical event for the development of HAM/TSP. Although several reports have shown that HTLV-1 has the ability to colonize within cells of the CNS, the precise mechanisms by which the virus traverses the BBB are poorly understood (27, 31). Enhanced adhesion and transmigration of HTLV-1-infected T lymphocytes with rat brain endothelial cells in vitro has suggested that trafficking of infected lymphocytes and macrophages across the BBB may be a mechanism of viral entry into the CNS (49). An increased expression of

adhesion molecules and proinflammatory cytokines has also been reported in spinal cord lesions of HAM/TSP patients (62). Proinflammatory cytokines have been shown to facilitate the transmigration of lymphocytes across the BBB by inducing the expression of adhesion molecules such as VCAM-1 on endothelial cells and lymphocytes (14, 47, 49). Indeed, elevated levels of TNF- α , IL-1 β , IL-1 α , and IL-6 detected in the cerebrospinal fluid of HAM/TSP patients suggest that these proinflammatory cytokines may play an important role in enhanced adhesion and transmigration of HTLV-1-infected lymphocytes into the CNS (28, 40, 57). Notably, selective chemokines have also been shown to enhance the migration of human immunodeficiency virus type 1 (HIV-1)-infected leukocytes across the BBB, suggesting similar mechanisms of infected cell migration in retroviral neuropathogenesis (12).

HTLV-1 infection has previously been detected in astrocytes and in infiltrating lymphocytes from the lesions of HAM/TSP patients (31, 38). We speculate that CNS resident cells such as astrocytes and oligodendrocytes may be infected *in vivo* and that Tax1-induced cytokine gene expression further mediates neurotoxicity. Astrocytes have previously been shown to support infection by mouse hepatitis virus (MHV), HIV-1, Theiler's murine encephalomyelitis virus (TMEV), and HTLV-1 (3, 5, 44, 55). Astrocytes interact with endothelial cells to form the BBB and may potentially also function as antigen-presenting cells (36). Our data show that HTLV-1 infection of astrocytes leads to a much more vigorous induction of proinflammatory cytokine gene expression pattern compared to HTLV-2. Oligodendrocytes have been shown to be cellular targets for neurotropic viruses, including JC virus, MHV, TMEV, and herpes simplex virus type 1 (13, 23, 43, 52). Viral infection of oligodendrocytes generally results in cytolysis, either as a direct result of viral infection or as a consequence of inflammatory and immune responses. Demyelination and paralysis is a common distinguishing feature of these infections, particularly in the case of infection with MHV and TMEV (13). We speculate that infection of oligodendrocytes with HTLV-1 results in Tax1-induced proinflammatory cytokine gene expression and acceleration of the demyelination process. Our data show that Tax1 expression results in the induction of TNF- α , TNF- β , and IL-6 in oligodendroglial cell lines and primary oligodendrogliomas. TNF- α , in particular, has previously been shown to be cytotoxic to oligodendrocytes and has been shown to sensitize oligodendrocytes to apoptosis in the spinal cord of HTLV-1-infected WKAH rats (21, 22).

TNF- α has been shown to adversely affect glutamate metabolism and expression of matrix metalloproteinase in astrocytes in transient contact with HTLV-1-infected T cells, and this has been hypothesized to result in the breakdown of the structural and functional integrity of the CNS (16, 17). The induction of matrix metalloproteinases has been implicated in extracellular matrix degradation and inflammation, and this can result in increased permeability of the BBB and facilitate enhanced migration of HTLV-1-infected cells into the CNS (18, 64). Interestingly, Tax transduction did not induce any IL-1 expression in oligodendrocytes, although IL-1 β expression was evident in primary oligodendrogliomas. Notably, chronic IL-1 β induction has been associated with breakdown of the BBB, enhanced recruitment of inflammatory cells, and the activation of T cells, microglial cells, and astrocytes (14). IL-1 β can also induce expression of neurotoxic mediators from glial cells resulting in inflammation and apoptosis (4). IL-6 has also been recently shown to mediate inflammation, demyelination, and cellular injury in the CNSs of transverse myelitis patients, suggesting its potential role in neuropathogenesis (24). Dysregulation of these proinflammatory cytokines may ultimately contribute to increased transmigration of HTLV-1-infected lymphocytes into the CNS, destruction of astrocytes and oligodendrocytes and manifestation of HAM/TSP.

Although genetically similar to HTLV-1, the association of HTLV-2 infection with neurodegenerative diseases is rare and controversial (2). It is not well understood what aspects of HTLV-2 biology contribute to its lack of pathogenicity since both viruses have similar epidemiology, modes of transmission and are highly genetically related. The vigorous induction of

proinflammatory cytokine gene expression after HTLV-1 infection of human astrocytes is consistent with previously published reports of induction of TNF- α and IL-1 α in HTLV-1-infected astrocytes (36, 53, 55). Interestingly, although HTLV-2 infection of human astrocytes also results in a similar pattern of proinflammatory cytokine gene expression, the overall induction levels were relatively weaker in comparison to levels detected with HTLV-1 infection. Similarly, although both Tax1 and Tax2 expression is sufficient for the induction of proinflammatory cytokines in astrocytes, Tax1 mediates a significantly more robust activation profile of these genes in comparison to Tax2. Interestingly, HTLV infection and Tax transduction failed to induce TGF- β in both astrocytes and oligodendrocytes, indicating that Tax specifically activates transcription of proinflammatory cytokine genes in neuroglial cells and does not globally activate cellular transcription. Previous studies have shown TGF- β to be an anti-inflammatory, antiapoptotic, and neuroprotective cytokine (45, 66, 68). It can be postulated that a threshold level of proinflammatory cytokine induction by Tax1, which is not achieved by Tax2, may be a distinguishing feature which manifests in the elevated neuropathogenesis after infection with HTLV-1.

Tax1 has generally been shown to protect lymphoid cells from apoptosis (37, 51, 60). Interestingly, our data demonstrate that Tax1 transduction in primary human astrocytomas sensitized these cells to apoptosis. Other investigators have previously reported that Tax1 can sensitize certain cell types to stress-induced apoptosis (8, 25, 59). Tax1-expressing rat fibroblasts have been shown to undergo apoptosis after serum deprivation (67). Tax1 has been speculated to downregulate the expression of *bcl-2*, resulting in apoptosis of oligodendrocytes in the spinal cord of HTLV-1-infected WKAH rats (22, 41, 58). Although we are in the process of determining whether Tax1 sensitizes oligodendrocytes to apoptosis, we speculate that synergy between induction of proinflammatory cytokines and apoptosis may result in either direct or bystander damage to both astrocytes and oligodendrocytes within the CNS of HAM/TSP patients.

The unique properties demonstrated by the chimeric Tax²²¹ construct suggest that the pattern of cytokine induction and sensitization to apoptosis may be mediated by domains located in the C-terminal 53 amino acids of Tax1, including PDZ-binding domain. Previous studies have shown that Tax1 C terminus enhances transformation, mediates the suppression of hematopoiesis in CD34⁺ cells, and activates expression of the cdk inhibitor genes p21^{cip1/waf1} and p27^{kip1} (11, 60, 61). Notably, Tax1 has been shown to dysregulate cell cycle in HTLV-1-infected T cells by binding to the precursor of IL-16, a cytokine encoding a PDZ domain that regulates cell growth and proliferation (65). The induction pattern displayed by Tax²²¹ suggests that the PDZ and/or the P/CAF binding domains located in the 3' C terminus of Tax1 may mediate the unique phenotypes displayed by Tax1 in neuronal cells and may be viral determinants of pathogenesis. Our data demonstrate that Tax1 C terminus clearly plays a role in transcriptional transactivation of cytokine gene expression and in the modulation of apoptosis in neuroglial cells. The generation of more precise Tax1 mutants will further illuminate the role of these domains in Tax1-mediated transactivation of host cyto-

kine gene expression and in mediating apoptosis in neuroglial cells.

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