Tax and Semaphorin 4D Released from Lymphocytes Infected with Human Lymphotropic Virus Type 1 and Their Effect on Neurite Growth

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Abstract

Human lymphotropic virus type 1 (HTLV-1) is a retrovirus causing HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurodegenerative central nervous system (CNS) axonopathy. This virus mainly infects CD4⁺ T lymphocytes without evidence of neuronal infection. Viral Tax, secreted from infected lymphocytes infiltrated in the CNS, is proposed to alter intracellular pathways related to axonal cytoskeleton dynamics, producing neurological damage. Previous reports showed a higher proteolytic release of soluble Semaphorin 4D (sSEMA-4D) from CD4⁺ T cells infected with HTLV-1. Soluble SEMA-4D binds to its receptor Plexin-B1, activating axonal growth collapse pathways in the CNS. In the current study, an increase was found in both SEMA-4D in CD4⁺ T cells and sSEMA-4D released to the culture medium of peripheral blood mononuclear cells (PBMCs) from HAM/TSP patients compared to asymptomatic carriers and healthy donors. After a 16-h culture, infected PBMCs showed significantly higher levels of CRMP-2 phosphorylated at Ser⁵²². The effect was blocked either with anti-Tax or anti-SEMA-4D antibodies. The interaction of Tax and sSEMA-4D was found in secreted medium of PBMCs in patients, which might be associated with a leading role of Tax with the SEMA-4D-Plexin-B1 signaling pathway. In infected PBMCs, the migratory response after transwell assay showed that sSEMA-4D responding cells were CD4⁺Tax⁺ T cells with a high CRMP-2 pSer⁵²² content. In the present study, the participation of Tax-sSEMA-4D in the reduction in neurite growth in PC12 cells produced by MT2 (HTLV-1-infected cell line) culture medium was observed. These results lead to the participation of plexins in the reported effects of infected lymphocytes on neuronal cells.

Introduction

HUMAN LYMPHOTROPIC VIRUS TYPE 1 (HTLV-1) is a retrovirus that may cause two diseases, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurodegenerative central axonopathy, and adult T cell leukemia (ATL), an aggressive neoplasia.^{1,2} This virus mainly infects CD4⁺ T lymphocytes; recent reports indicate that a subpopulation of CD4⁺CD25⁺CCR4⁺ cells is infected to a greater extent.^{3,4} Monocytes, CD8⁺ T lymphocytes, B cells, and dendritic cells are also infected to a lesser extent.^{1,5–7}

Worldwide it is estimated that 15–20 million people are infected with HTLV-1, but only 3–5% of the infected subjects develop HAM/TSP.^{8,9} Histologically, this disease is

considered a central nervous system (CNS) axonopathy caused by alterations in axoplasmic transport, producing a degenerative process in the axon without affecting the cell body.¹⁰ Spinal cord atrophy in HAM/TSP is observed in the thoracolumbar cord followed by cross-sectional studies.^{10,11} No evidence of neuron infection with HTLV-1 has been detected so far.^{5,12} HTLV-1-infected peripheral T cells cross the blood–brain barrier; thus neurons of the CNS are in contact with secreted viral products, such as Tax and other secreted T cell proteins. Infected astrocytes, lymphocytes, and endothelial cells have been detected in the spinal cord, including periventricular areas of the blood barrier, sub-arachnoid space, and thoracic and lumbar regions, implying direct contact between infected cells and CNS cells.^{13–15} Tax

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protein secreted from CNS-infiltrating infected lymphocytes might be involved in the mechanisms of axonopathy of paraparesis, altering intracellular pathways related to axonal cytoskeletal dynamics.^{16–18}

Our group has recently reported that the Tax secretory pathway in peripheral blood mononuclear cells (PBMCs) is mediated by the classical ER-Golgi.¹⁹ Only 60% of HAM/TSP clinically diagnosed patients are seropositive for HTLV-1 when using the traditional ELISA test based on surface antigen recognition.²⁰ Nevertheless, both Tax protein and tax gene expression have been detected in all patients with progressive spastic paraparesis—seropositive or seronegative.²¹ In these cases, patients may have a truncated version of the provirus not expressing surface antigens. These observations and the identification of Tax in cerebrospinal fluid (CSF) have led us to suggest a major role for Tax in the pathogenesis.²²

Our group has reported that Tax secreted from an HTLV-1-infected human T cell line (MT2) produced retraction in neuroblastoma cells, SH-SY5Y.²³ This effect could be mediated by Tax as well as by other proteins released from HTLV-1-infected lymphocytes such as a proteolytically shed form of Semaphorin 4D, SEMA-4D (150-kDa transmembrane glycoprotein), called soluble Semaphorin 4D, sSEMA-4D. This soluble semaphorin is a bioactive soluble form of 120-kDa that upon binding to its neuronal receptor Plexin B1 induces growth cone collapse.^{24–28}

SEMA-4D is expressed at low levels in resting T cells, B cells, macrophages, NK cells, and dendritic cells, and upon activation of these cells SEMA-4D is up-regulated.^{29,30} SEMA-4D shedding is blocked by matrix metalloproteinase (MMP) inhibitors, with ADAM17 and MT1-MMP (membrane-type-1 matrix metalloproteinase) involved.^{31,32}

Human multipotent neural precursors or primary oligodendrocytes from rat brain exposed to T cells—chronically activated by HTLV-1-expressing sSEMA-4D—induce apoptosis and neurite extension collapse, respectively.^{33,34} In HAM/TSP patients, sSEMA-4D was detected in CSF samples, and SEMA-4D⁺ T cells were found in demyelinated spinal cord specimens.³³ sSEMA-4D causes neurite retraction in PC12 cells and axonal growth cone collapse in primary hippocampal neurons.^{25,35} The downstream signaling of the receptor Plexin-B1 includes both PI3 kinase and CRMP-2 (collapsin response mediator protein) inactivation

and Akt and GSK-3 β dephosphorylation mediated by R-Ras Gap activity, ultimately producing growth cone collapse.^{26,27,36,37} CRMP-2 protein is not only involved in axon collapse associated with semaphorin signal transductions but is also related to T cell migration in activated lymphocytes.³⁸ CRMP-2 plays a key role in cytoskeleton reorganization, controlling infected lymphocyte migration and then targeting to the brain; this function is regulated by its phosphorylation in specific sites, such as Tyr⁴⁷⁹.^{39,40} Recruitment of infected lymphocytes into the CNS is essential for the development of HAM/TSP induced by HTLV-1.^{40,41} A hypothetical model of the role of Tax and sSEMA-4D on signaling pathways resulting in neurite degeneration is presented in Fig. 1.

The current study focuses on Tax, sSEMA-4D, and CRMP-2 in PBMCs isolated from HAM/TSP patients. An increase in the SEMA-4D and sSEMA-4D levels was detected in HAM/TSP patients compared to asymptomatic carriers and healthy donors. In patients, we detected higher levels of MT1-MMP, metalloproteinase involved in SEMA-4D shedding. An interaction was found between sSEMA-4D and Tax in the culture medium of PBMCs isolated from HAM/TSP patients. CRMP-2 phosphorylated at S⁵²² was shown to be significantly increased in patients compared to asymptomatic carriers. The migratory response in SEMA-4D responding cells from HAM/TSP patients was associated with CD4⁺Tax⁺ T cells showing an increase in CRMP-2 pSer⁵²². Finally, we evaluated the participation of these proteins in the reduction of PC12 cell neurite growth produced by MT2 culture medium.

Materials and Methods

HAM/TSP patients, asymptomatic carriers, and healthy control subjects

All the experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. The University of Chile Ethics Committee has approved the procedure and all individuals gave informed written consent. All HAM/TSP patients fulfilled the criteria of gait commitment according to The World Health Organization. EDTA-treated blood was obtained from 53 HAM/TSP patients, 19 asymptomatic carriers, and 8 healthy donors.

FIG. 1. Proposed hypothetical model. Human lymphotropic virus type 1 (HTLV-1)-infected lymphocytes have increased CRMP-2 phosphorylation facilitating their migration and recruitment into the central nervous system (CNS). The higher MT1-MMP expression increases the cleavage of Semaphorin 4D (SEMA-4D) from the membrane releasing its soluble form soluble Semaphorin 4D (sSEMA-4D) that interacts with the secreted viral protein Tax. Tax-sSEMA-4D may bind Plexin-B1, impairing axon growth.

Isolation and culture of PBMCs

PBMCs were obtained from 10 ml of EDTA-treated blood by Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque Plus Amersham Pharmacia Biotech Catalog No. 17-1440-03) and then were washed three times with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 100 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2). We collected 7–10 × 10⁶ PBMCs from each sample, which were cultured for 24 h in RPMI 1640 + GlutaMAX (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% iFBS (inactivated fetal bovine serum, Thermo Fisher Scientific). To inhibit the action of CD8⁺ cytotoxic T lymphocytes, 20 nM concanamycin A was added to the culture media (Sigma-Aldrich, St Louis, MO) as performed by Alberti *et al.* (2000).⁴² The counting of viable and nonviable cells was measured by 0.4% trypan blue exclusion dye (Sigma-Aldrich). This method showed more than 95% viable PBMCs, which agrees with the manufacturer's statement (Amersham Bioesciences). To obtain culture media, cells were separated by centrifugation at 400 × g, 5 min at 4°C, collecting the supernatant (culture medium) and the pellet fraction (cells).

MT2 and K562 cell cultures

Cultures of MT2 (HTLV-1-infected cell line) and K562 (control noninfected cell line) were maintained in RPMI 1640 + Glutamax and 10% iFBS. To obtain media with secreted products of both cell lines, in each case, 1 × 10⁶ cells/ml was cultured in RPMI 1640 + Glutamax and 0.2% iFBS for 7 days. Then cells were centrifuged at 400 × g for 5 min at 4°C collecting the supernatant (culture medium) and the cells in the pellet fraction.

Tax and sSEMA-4D immunodepletion of MT2 culture medium was done using the AminoLinkPlus Immobilization Kit (Pierce Biotechnology, Rockford, IL). Antibodies either against Tax (Covalab, Villeurbanne, France, Catalog No. mab0022) or SEMA-4D (Santa Cruz Biotechnology, Santa Cruz, CA, Catalog No. sc-79404) were immobilized following the manufacturer's instructions. MT2 culture medium was applied and unbound sample was collected by centrifugation.

Cell lysis and protein determination

After cell culture and centrifugation as described above, MT2 cells, K562 cells, or PBMC pellets were washed five times with phosphate-buffered saline and the last pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 10 mM N-ethylmaleimide, 0.2 mM Na₃VO₄, and 0.1 mM PMSF, pH 7.5). Lysis was done with 2 × 10⁷ cells per 500 µl of lysis buffer (on ice) with gently shaking. Cell debris was pelleted by centrifugation at 16,000 × g for 5 min at 4°C. The lysate was kept at –20°C. Protein determination of cell lysates was done using the Micro BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions.

Western blot analysis

SDS/PAGE under reducing conditions was performed with 10% polyacrylamide gels. Portions of 50 µg protein of cell lysate or 20 µl of PBMC culture media or 50 µl of MT2 or K562 culture media were used. The buffer for electrotransfer to nitrocellulose membranes (Bio-Rad Laboratories Ltd,

Hercules, CA) contained 25 mM Tris-HCl, 192 mM glycine, and 20% methanol, and electrotransfer was done at a total of 600 mA at 4°C. After electrotransfer, membranes were blocked for 20 min at room temperature with 6% nonfat milk dissolved in TBS-T (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.6), then incubated overnight at 4°C or 2 h at room temperature with different primary antibodies at the appropriate dilution in TBS-T.

The following monoclonal antibodies were used: anti-Tax antibody (Covalab, diluted 1:1,000) in the experiments of Fig. 3C, anti-Tax antibody from HTLV-1 hybridoma 168A51-2 culture supernatant (NIH, AIDS Reagent Program, Germantown, MD; Catalog No. 1316, diluted 1:1 with TBS-T) in the other experiments, and anti-GAPDH antibody (Sigma-Aldrich, St Louis, MO; Catalog No. G8795 diluted 1:20,000).

The following polyclonal antibodies were used: anti-SEMA-4D antibody (Santa Cruz Biotechnology, diluted 1:5,000), anti-MT1-MMP antibody (Catalog No. AB815, diluted 1:1,000), anti-MMP-9 antibody (Millipore, Catalog No. MAB3309, diluted 1:1000), anti-CRMP-2 total (Sigma-Aldrich, Catalog No. C2993, diluted 1:50,000), and anti-CRMP-2 pS⁵²² (ECM Biosciences, Versailles, KY; Catalog No. CP2191, diluted 1:2,000). After 5 washings with TBS-T (5 min each), membranes were incubated with the appropriate secondary antibody for 1 h at room temperature.

The following secondary antibodies were used: ImmunoPure Goat Anti-Rabbit IgG (H+L), peroxidase conjugated (Pierce, Rockford, IL, diluted 1:500,000), ImmunoPure Goat Anti-Mouse IgG (H+L), peroxidase conjugated (Pierce, diluted 1:500,000), and Rabbit anti-Goat IgG (H+L), peroxidase conjugate (Pierce, diluted 1:500,000). When anti-Tax antibody from the HTLV-1 hybridoma 168A51-2 culture supernatant was used as the primary antibody, Donkey anti-Mouse IgG (H+L) cross-adsorbed, peroxidase conjugate (Pierce, diluted 1:250) was the secondary antibody employed. After five washings with TBS-T (5 min each), the antibody interaction was visualized with enhanced Super Signal West Femto Maximum Sensitivity Substrate Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

When Donkey anti-Mouse IgG (H+L) Cross Adsorbed, peroxidase conjugate was used as the secondary antibody the EZ-ECL Chemiluminescence Detection Kit for HRP (Biological Industries, Kibbutz Beit Haemek, Israel) was employed. X-Ray films (CL-Xposure film, Pierce) were exposed for varying times. Control experiments without primary antibodies (with only secondary antibodies) did not give any chemiluminescent signal. To analyze the same membrane with a different antibody, stripping was performed washing twice with 200 mM glycine, 3.5 mM SDS, and 10% Tween-20 at pH 2.2 (10 min each). Membranes were then blocked and Western blot was performed as described above using another primary antibody. Quantification of blots was carried out by scanning films using the Uni-Scan-It Automated Digitizing System.

PBMCs flow cytometry

PBMCs (300,000 cells per well) for flow cytometry were cultured 14 h in RPMI 1640 (Gibco, Paisley, UK) with 10% iFBS and 20 nM concanamycin A.⁴² Cells were harvested and stained with fluorophore-conjugated antibodies from eBiosciences (San Diego, CA): CD4-PE (Catalog No.

53-0048-71, diluted 1:34), CD4-FITC (Catalog No. 53-0048-73, diluted 1:25), and CD100/SEMA-4D-FITC (Catalog No. 53-1009-73, diluted 1:100). Tax-APC was generously donated by Dr Tanaka (diluted 1:100). Matched isotype controls of the respective antibodies were used at the same dilution. We performed two-color flow cytometry in a FACS-CANTO instrument (Beckton Dickinson). WinMDI 2.9 software was used for data analysis.

Effect of Tax in sSEMA-4D levels in PBMC culture medium of HAM/TSP patients

PBMCs cultured in RPMI 1640 + GlutaMAX, 10% iFBS, and 20 nM concanamycin A were treated with or without anti-Tax (Covalab, Catalog No. mab0022 diluted 1:100) and the levels of sSEMA-4D were determined by Western blot (as described above) for 2.5 h every 30 min.

Coimmunoprecipitation assay

A pool of 10 ml of PBMC culture media from five different HAM/TSP patients was used for immunoprecipitation using anti-SEMA4D antibody (27 μ l). As control anti-MMP-9 antibody (20 μ l) was used. Antibodies were covalently bound to the resin of the AminoLink Plus Immobilization Kit (Pierce). Antibody binding, sample application, and protein elution were done according to the manufacturer's instructions. Proteins eluted from the matrix were subjected to SDS/PAGE and Western blot.

Transwell migration assay

PBMCs from three different HAM/TSP patients were cultured for 16 h similar to the manner described above. Portions containing 1×10^6 cells were placed on the upper layer of the 3- μ m-diameter semipermeable membrane allowing lymphocyte migration, in RPMI supplemented with 5% iFBS and 20 mM concanamycin A. In the lower chamber 1 ng/ml recombinant sSEMA-4D was included. Migration through the membrane was followed up to 6 h at 37°C. Portions of 10 μ l were removed from the lower chamber for cell counting under an optic microscope. Data were expressed as the increase in the number of cells that migrated with respect to the control condition (the medium without sSEMA4-D). Cells that migrated were characterized by flow cytometry measuring the CD4⁺Tax⁺ population. In a parallel experiment also included was the addition in the upper chamber of PBMCs from a healthy donor, adding sSEMA-4D in the lower chamber. In addition, in the cell lysate the level of CRMP-2 p⁵²² was determined by Western blot.

PC12 cell culture, differentiation and treatment with MT2 culture medium

Undifferentiated PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM HEPES Modification and High Glucose, Sigma-Aldrich), 6% iFBS, and 6% horse serum (HS, Catalog No. 16050, Invitrogen, Eugene, OR). Differentiation to neuronal type was initiated with the addition of 50 ng/ml of nerve growth factor (NGF; Alomone Labs, Jerusalem, Israel) to PC12 cells seeded at a density of 45,000 cells per cm² in DMEM 6% iFSB and 6% HS. Cells were then differentiated during 3 days gradually decreasing the serum concentration as follows: culture with DMEM 4% iFBS, 4%

HS, 50 ng/ml NGF during the first day; with DMEM 2% iFBS, 2% HS, 50 ng/ml NGF during the second day; and with DMEM 1% iFBS, 1% HS, 50 ng/ml NGF during the third day.

During differentiation, PC12 cells were cultured with 1/8 of MT2 or K562 culture media that included the vehicle comprising RPMI 1640 + Glutamax and 0.2% FBS. Tax and sSEMA-4D neutralization in MT2 media was done daily adding either anti-Tax antibody (isolated from HTLV-1 Tax hybridoma 168A51-2 ascitic fluid) or anti-SEMA-4D antibody (both antibodies at dilution of 1:100 in the differentiation medium) daily to PC12 differentiation medium. Control experiments with isotype-matched irrelevant antibodies (anti-Gizzerosine) were also done at 1:100 dilutions.

Neurite outgrowth evaluation

Cells were observed under a phase-contrast microscope using a 20 $\times$ objective. Images were directly captured as black-and-white digital micrographs. Eight areas of PC12 cells were randomly acquired, measuring between 20 and 70 neurites per field. Neurite length was measured using the NIH ImageJ-1.38d program plug in NeuronJ. The neurite length corresponds to the net extension away from the cell body (from the margin of the cell body to its terminus). Only neurites with no secondary prolongations were considered.

Statistical analysis

All statistical analysis in these studies was performed with the Statistical Package of the Social Sciences (SPSS 17). Values are mean $\pm$ SEM of at least three independent experiments. Group comparison was done using ANOVA when data showed a normal distribution or Kruskal-Wallis when the distribution was not normal. In cases in which the population means are not equal according to ANOVA, the multiple comparisons Post Hoc test of Tukey was set up to determine whether pairs of means are different. Data were significantly different if $p < 0.05$ in all cases.

Results

Tax, SEMA-4D, and MT1-MMP in cell lysates and in PBMC culture medium

The occurrence of these proteins was initially studied in PBMCs of three HAM/TSP patients and three healthy donors by Western blot. Representative Western blots of Tax (57 kDa), SEMA-4D (150 kDa), and sSEMA-4D (120 kDa) in both culture media of PBMCs and cell lysates are showed in Fig. 2. Ubiquitinated Tax with a molecular mass of 57 kDa was detected in both cell lysates and culture media of infected PBMCs as previously reported.¹⁹ PBMC lysates and culture media from healthy donors did not show Tax (Fig. 2).

Comparing HAM/TSP samples of patients versus healthy donors, we found higher levels of sSEMA-4D in the culture medium (Fig 2A) and SEMA 4-D and MT1-MMP in cell lysates (Fig. 2A and B). These results led us to measure SEMA-4D in a larger population of HAM/TSP patients.

Analysis of SEMA-4D in PBMCs from HAM/TSP patients, asymptomatic carriers, and healthy donors

Cohorts including PBMCs from 53 HAM/TSP patients, 19 asymptomatic carriers, and 8 healthy donors were analyzed

FIG. 2. Western blot analysis of the expression of Tax, SEMA-4D, and MT1-MMP in peripheral blood mononuclear cells (PBMCs) of an HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patient. (A) Detection in 20 μ l of PBMC culture medium and (B) in PBMC lysates. GAPDH is used as loading control. Lane HAM, HAM/TSP, patient sample; lane Control, healthy donor sample ($n=3$).

FIG. 3. Flow cytometry analysis of the CD4⁺SEMA-4D⁺ population in HAM/TSP patients, asymptomatic carriers, and healthy donors and SEMA-4D proteolytically shed from PBMCs. (A) Dot-plot representation of selected lymphocyte population of PBMCs, (B) CD4⁺SEMA-4D⁺ population in an HAM/TSP patient, (C) CD4⁺SEMA-4D⁺ population in a healthy donor, and (D) CD4⁺SEMA-4D⁺ population in an asymptomatic carrier. (E) Data analysis of the percentage of CD4⁺SEMA-4D⁺ cells obtained by flow cytometry shows statistical differences between HAM/TSP patients and both asymptomatic carriers (carriers) and healthy donors (controls). (F) sSEMA-4D analysis by Western blot also shows significant differences between HAM/TSP patients and the other two groups. Statistical significance with * $p < 0.05$.

for CD4⁺SEMA-4D⁺ markers by flow cytometry. Representative dot-plots are shown in Fig. 3A–D. A significant increase of the membrane-bound SEMA-4D was found in HAM/TSP samples compared with the other two groups, with no differences between asymptomatic carriers and controls (Fig. 3E). These results agree with those of sSEMA-4D in PBMC culture media by Western blot (49 HAM/TSP patients, 13 asymptomatic carriers, and 7 healthy donors). Accordingly, we found a significant increase of this soluble form of semaphorin only in HAM/TSP patient samples compared with those of asymptomatic carriers and healthy donors (Fig. 3F). These results suggest that sSEMA-4D released from infected PBMCs in the CNS plays a relevant role in the development of HAM/TSP.

Tax and sSEMA-4D release from PBMCs of HAM/TSP patients and their interaction

Soluble SEMA-4D occurs in culture media due to the cleavage of an extracellular portion of the membrane-bound SEMA-4D protein.³² Extracellular Tax could be involved in sSEMA-4D release from infected T cells. To determine the effect of extracellular Tax, PBMCs from HAM/TSP patients were cultured in the presence of anti-Tax antibody in the culture medium. The inclusion of anti-Tax antibodies reduced the release of sSEMA-4D into the culture medium as shown in Fig. 4A (upper panel) and Fig. 4B. Addition of an irrelevant isotype-matched antibody (anti-Gizzerosine) did not reduce the extracellular levels of sSEMA-4D (data not shown). The blockage of Tax effects by anti-Tax antibody supports the involvement of this protein in the release of sSEMA-4D into the culture medium of infected lymphocytes.

FIG. 4. Tax effect on SEMA-4D shedding and interaction of both proteins in PBMC culture media from HAM/TSP patients. (A) Representative Western blot of sSEMA-4D using PBMC culture medium from an HAM/TSP patient cultured with (upper panel) or without (lower panel) anti-Tax antibody. Lanes 1, 2, 3, 4, 5, and 6 represent 0, 0.5, 1, 1.5, 2, and 2.5 h, respectively, after anti-Tax antibody addition to the culture medium. (B) Analysis of sSEMA-4D data expressed in arbitrary units (AU) of pixel intensity ($n=3$). Statistical significance with $*p<0.05$; $**p<0.01$; $***p<0.001$ when each time is compared with the initial time. (C) Coimmunoprecipitation of sSEMA-4D and Tax from PBMC culture medium from an HAM/TSP patient detected by Western blot (sSEMA-4D in lane 1 and Tax in lane 2).

The interaction of Tax and sSEMA-4D in culture medium was also evaluated. The culture medium of PBMCs from HAM/TSP patients was submitted to immunoprecipitation with antibodies against SEMA-4D. Viral protein Tax was found in the immunoprecipitate (Fig. 4C). Immunoprecipitation with an irrelevant antibody (metalloproteinase 9) was used as negative control where Tax was not found in the immunoprecipitate (data not shown). This coimmunoprecipitation provides evidence of the existence of the interaction of Tax and sSEMA-4D in the extracellular medium, suggesting that Tax secretion from infected lymphocytes could directly assist sSEMA-4D release.

CRMP-2 phosphorylation at Ser⁵²² in HTLV-1-infected lymphocytes

We studied the phosphorylation of CRMP-2 at Ser⁵²², which contributes to impaired neurogenesis by disturbing microtubule integrity in maturing neurons.⁴³ CRMP-2 pSer⁵²² was found by Western blot in PBMC lysates from 27 patients and was compared to 15 asymptomatic carriers (Fig. 5A). These results suggest an increase in Cdk5 activity, the enzyme responsible for this phosphorylation.⁴³ To the best of our knowledge, phosphorylation of the Ser⁵²² residue has not been previously reported in HTLV-1-infected lymphocytes. This posttranslational modification could differentially regulate CRMP-2 function in PBMCs from HAM/TSP patients and asymptomatic carriers.

Additionally, Tax and sSEMA-4D could be involved in the mechanism of CRMP-2 phosphorylation at Ser⁵²² in PBMCs from HAM/TSP patients. To address the effect of Tax and sSEMA-4D in CRMP-2 phosphorylation, PBMCs from HAM/TSP were cultured with antibodies against either Tax or sSEMA-4D. Subsequently, CRMP-2 phosphorylation at Ser⁵²² was evaluated on lysates of cultured PBMCs at different times. A significant reduction in phosphorylation was observed in both cases up to 1.5 h of culture (Fig. 5B and C).

We also evaluated the effect of culture medium of infected lymphocytes on CRMP-2 phosphorylation in noninfected PBMCs (from healthy donors). Since no phosphorylated CRMP-2 was found in PBMCs from noninfected subjects, a modification of Ser⁵²² may be occurring because of products secreted by infected PBMCs. Figure 5D and E shows Ser⁵²² phosphorylated CRMP-2 in PBMCs from healthy donors when cultured with medium from infected PBMCs. Additionally, when culture medium was either Tax or sSEMA-4D immunodepleted, CRMP-2 pSer⁵²² levels decreased compared with complete culture medium (Fig. 5D and E). It is worth noting that the degree of phosphorylation reached in these lymphocytes from healthy donors was about the same magnitude as that observed in patient's lymphocytes (Fig. 5A). Data suggest that both Tax and sSEMA-4D would be necessary to enhance CRMP-2 phosphorylation at Ser⁵²². Extracellular Tax may activate the signal transduction pathway involving CRMP-2 regulation, and this effect could be mediated by sSEMA-4D via the Plexin B1 receptor.

Migration response to sSEMA-4D of lymphocytes from HAM/TSP patients

To evaluate the migration response of HAM/TSP patient's PBMCs, transmigration experiments were performed using the transwell system. The lower chamber was settled with 1 ng/ml

FIG. 5. CRMP-2 phosphorylation at Ser⁵²² in infected lymphocytes and effect of culture medium containing Tax and sSEMA-4D on non-infected lymphocytes. (A) CRMP-2 phosphorylation levels in PBMCs from HAM/TSP patients and asymptomatic carriers. Data are expressed as arbitrary units (AU) of pixel intensity of the ratio CRMP-2 pS⁵²²/total CRMP-2 obtained by Western blot. Effect of the inclusion of anti-sSEMA-4D (B) or anti-Tax (C) antibody in the culture medium of PBMCs from an HAM/TSP patient. (D) Representative Western blot of CRMP-2 pS⁵²² (upper panel) and total CRMP-2 (lower panel). PBMC lysates from healthy controls were cultured in standard conditions (lanes 1, 2), including PBMC culture medium from HAM/TSP patients (lanes 4, 5), including Tax immunodepleted culture medium (lanes 6, 8) or SEMA-4D immunodepleted culture medium (lanes 3, 7). (E) Data analysis of the CRMP-2 phosphorylation of PBMC lysates treated as described in (D) showing a significant reduction in both immunodepleted conditions. Statistical significance with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of recombinant sSEMA-4D and the transmigration pattern was followed up to 6 h by flow cytometry. The cell number in the lower chamber with sSEMA-4D increased in a time-dependent fashion compared with the control condition (absence of sSEMA-4D), which remained unaltered during the experiment's time course (Fig. 6A). The transmigration was expressed as the times of changes of the number of cells with respect to the control condition. The samples placed initially in the upper chamber contained around 3% of CD4⁺Tax⁺ T cells, similar to a previous report from our group. Accordingly, the migrated lymphocyte population became enriched in CD4⁺Tax⁺ T cells throughout the study compared with the control, being up to 22% of the PBMC population at 6 h (Fig. 6B). Therefore, HTLV-1-infected CD4⁺ T cells from HAM/TSP patients migrate in response to sSEMA-4D.

Additionally, in a parallel experiment CRMP-2 phosphorylation levels were evaluated in the lower chamber population by Western blot. In this experiment the control employed was a healthy donor sample (noninfected control) placed in the upper chamber, adding sSEMA-4D in the lower chamber. The results shown in Fig. 6C indicate higher amounts of CRMP-2 pSer⁵²² in cell lysates from HAM/TSP patient's

PBMCs that migrated to the lower chamber compared with those from the upper chamber. In the control condition analysis by Western blot of the sample in the lower chamber was not possible because of the limited number of cells (around 70 cells per μ l); thus this analysis was done in cell lysates from the upper chamber (Fig. 6C). This variation in CRMP-2 phosphorylation between the studied populations could be related to the differences in migration patterns observed.

Tax and sSEMA-4D decreases neurite growth rate of PC12 cells

Ethical considerations limit the acquisition of HAM/TSP patient blood samples; thus, we used, for these studies, culture medium of MT2 cells (chronically HTLV-1-infected lymphocytes) and medium of K562 cells as control (noninfected cells). We identified sSEMA-4D (120 kDa) and Tax (40 kDa) in the culture medium of MT2 cells, but not in K562 culture medium (Fig. 7A). We previously reported that secreted Tax by MT2 cells lacks ubiquitin modification; therefore it migrates as a 40-kDa protein in Western blot analysis.¹⁹ An increase in both SEMA-4D and MT1-MMP

FIG. 6. Transmigration analysis of lymphocytes in response to sSEMA-4D. **(A)** Times of changes in the number of transmigrated cells from the control condition in response to 1.0 ng/ml of sSEMA-4D after 1 and 6 h of the assay. The control does not include sSEMA-4D in the lower chamber. **(B)** Flow cytometry analysis of the PBMC populations that migrated after 1 and 6 h. **(C)** Data analysis of CRMP-2 phosphorylation observed in the lower chamber and upper chamber in response to sSEMA-4D. Control condition corresponds to the values found in the upper chamber of the transwell system using PBMCs from a healthy donor ($n=4$). Data are represented as arbitrary units (AU) of pixel intensity of the ratio CRMP-2 pS⁵²²/total CRMP-2 obtained by Western blot. Statistical significance with $**p<0.01$; $***p<0.001$.

was found in MT2 lysates with respect to K562 lysates (Fig. 7B). These results are similar to those obtained using PBMCs from HAM/TSP patients and healthy donors.

In unpublished results we found that the addition of MT2 culture medium to PC12 cells during neuronal differentiation decreases the rate of neurite growth. The involvement of Tax and sSEMA-4D in this effect was followed by the addition of either anti-Tax or anti-SEMA-4D antibodies to the MT2 culture medium as blocking agents of their activities. As control we added the isotype matched irrelevant antibody (anti-Gizerosine antibody). Representative microphotographs of the effect of MT2 cell culture medium on PC12 neurite extensions are shown in Fig. 8A. A reduced neurite length of PC12 cells was observed in the presence of MT2 culture medium with or without the irrelevant antibody, compared either with the control (K562 culture medium) or

with MT2 culture medium with anti-Tax antibody (Fig. 8B). The neutralizing effect of the anti-Tax antibody indicates Tax involvement in neurite length reduction of PC12 cells during their differentiation. Experiments testing the addition of anti-SEMA-4D antibody on PC12 neurite length also showed a blocking effect of the MT2 medium, thereby indicating the involvement of sSEMA-4D too (Fig. 8). Altogether, these results indicate the participation of both Tax and sSEMA-4D in the reduced neurite length of PC12 cells.

Discussion

Brain-blood barrier disruption in HAM/TSP has been suggested by observations of perivascular cuffing with lymphocyte and macrophage infiltrates.¹⁷ Alterations in the barrier increase the permeability facilitating lymphocyte passage

FIG. 7. Detection of Tax, SEMA-4D, and MT1-MMP in MT2 cells and in their culture medium. **(A)** Detection in 20 μ l of infected MT2 cell culture medium and **(B)** in 50 μ g MT2 cell lysate. GAPDH was used as loading control.

FIG. 8. Tax effect on neurite length extension in PC12 cells. (A) Representative microphotographs of PC12 cells after 3 days of differentiation incubated with MT2 culture medium, K562 culture medium, MT2 medium with anti-Tax antibody, MT2 medium with anti-SEMA-4D antibody, and MT2 medium with an irrelevant antibody as control. (B) Data analysis of the neurite length expressed as mean values of $\mu\text{m} \pm \text{SEM}$, showing a significantly lower values of neurite length in the presence of Tax protein and sSEMA-4D. Significance with respect to MT2 condition with $*p < 0.05$.

through the monolayer of endothelial cells. The increase of adhesion molecules and the increase in metalloproteinase expression are also two viral strategies to facilitate the entry of infected lymphocytes into the CNS.⁴⁴ In some viral-induced neuroinflammation, high CRMP-2 expression in peripheral lymphocytes is involved in T cell polarization and migration to the brain.^{41,45} CRMP-2 levels are modulated by Tax as shown in a Jurkat T cell line transfected with Tax.⁴⁰ CRMP-2 is part of the PI3K/Akt/GSK-3 β signaling pathway essential for cell migration.²⁷ HTLV-1 infection activates this pathway, facilitating the migratory activity of infected T cells.⁴⁶

HTLV-1-infected T cell lines showed an increase in CRMP-2 pTyr⁴⁷⁹ and, to a greater extent, phosphorylation at Ser⁵²² and Thr⁵⁰⁹.⁴⁰ However, Tax knockdown experiments in Tax-transfected Jurkat cells suggest that Tax is not associated with these posttranslational modifications. CRMP-2 in T cells binds cytoskeleton, modulating the conversion from a semirigid to a highly deformable form, thus facilitating transendothelial migration and crawling within CNS.⁴⁵ In Jurkat T cells, CRMP-2 phosphorylated at Tyr⁴⁷⁹ displayed a greater ability to travel on mouse hippocampal organotypic culture.³⁹ In HAM/TSP patients, this protein exhibits a significantly elevated gene expression in the cell subset with activation markers CD69⁺ and HLA-DR⁺ (activated lymphocytes), with CD45RO⁺ (activated memory T cells marker), and with VLA-4⁺ (cell migration marker).³⁸

The Tax effect on CRMP-2 phosphorylation at Ser⁵²² was currently studied in infected human T cells; anti-Tax antibodies significantly lowered phosphorylation at Ser⁵²² (Fig.

4C). This phosphorylable residue is a substrate of Cdk5.⁴³ SEMA-4D is also involved in CRMP-2 phosphorylation at Ser⁵²² as the latter is reduced in the presence of anti-sSEMA-4D antibodies (Fig. 5B). Significantly higher levels of CRMP-2 pSer⁵²² were detected in PBMCs from HAM/TSP patients compared to asymptomatic carriers (Fig. 5A).

The neuroimmune semaphorin SEMA-4D exhibits a dual role in the immune and nervous system.²⁹ Both the increased level of sSEMA-4D released from PBMCs of HAM/TSP patients and the interaction of Tax with this semaphorin suggest that Tax secreted by HTLV-1-infected lymphocytes might play a role in the pathology. The significantly higher levels of sSEMA-4D in PBMC culture medium of HAM/TSP patients can be explained by an increase in matrix metalloproteinase expression/activity. The imbalance between the activities of MMPs and their inhibitors TIMPs (tissue inhibitors of matrix metalloproteinases) could also be responsible for the higher levels of sSEMA-4D in HAM/TSP samples. The Tax protein could be involved in either of these molecular changes. A significant increase in MMP-9 and MMP-3 levels and a mild increase in TIMP-2, TIMP-3, and TIMP-4 levels were previously detected by our group in CSF of HAM/TSP patients.⁴⁷ Additional studies should be performed on patients to determine the participation of TIMPs in plasma samples. In the present study, the active form of MT1-MMP associated with SEMA-4D cleavage was increased in HAM/TSP patients compared to healthy donors. The elevated MT1-MMP protein may account for the higher level of the SEMA-4D-soluble form.

The larger population of CD4⁺SEMA-4D⁺ cells and the greater amount of sSEMA-4D released in PBMCs from HAM/TSP patients compared to asymptomatic carriers and healthy donors suggest that sSEMA-4D is one of the proteins responsible for the progression of paraparesis. The incubation of PBMCs from HAM/TSP patients with anti-Tax antibodies significantly decreased sSEMA-4D release, indicating that the Tax secreted by HTLV-1-infected lymphocytes could increase sSEMA-4D (Fig. 4A and B). In addition to higher proteolytic shedding, the increased level of sSEMA-4D could be associated either with an enhancement of SEMA-4D expression or with an increase in sSEMA-4D half-life when complexed with Tax. In HAM/TSP patients, sSEMA-4D was detected in CSF samples and SEMA-4D⁺ T cells were also found in demyelinated spinal cord specimens.³³

The transwell experiment showed that the SEMA-4D attraction of PBMCs from HAM/TSP patients is associated with CD4⁺Tax⁺ cells containing a higher ratio of CRMP-2 pSer⁵²²/total CRMP-2. This result agrees with previous reports on HTLV-1-infected T cell migration toward cytokines.^{39,40}

We investigated the role of Tax and sSEMA-4D on neurite growth in PC-12 cells, expressing Plexin-B1. PC12 cells cocultured with 293T cells (expressing SEMA-4D) induced neurite retraction.³⁵ The onset of neuritogenesis depends largely on the dynamics of the actin cytoskeleton, regulated by several actin-binding proteins.⁴⁸ Axon elongation is positively regulated via Rac/Cdc42 and negatively regulated via the RhoA pathway. NGF-induced neurite outgrowth requires both the activation of the Ras/Raf/MEK/ERK and PI3K/Akt pathways.⁴⁹ MT2 culture medium was used instead of the culture medium of HAM/TSP patients because of bioethical regulations that impose a limitation on acquiring these samples. Figure 7 shows Tax expression in MT2 cells, its secretion to the culture medium, and the increased levels of MT1-MMP, SEMA-4D, and sSEMA-4D in MT2 cells compared to K562 cells (noninfected cell line). These results are similar to those using PBMC patient samples, validating the utilization of the MT2 cell line.

In our *in vitro* model, blocking Tax and sSEMA-4D with their respective antibodies led us to confirm their major role in reducing the rate of neurite elongation during NGF differentiation. The interaction of Tax and sSEMA-4D suggests that sSEMA-4D–Tax may be an activating complex of the receptor. Another relevant result of this study is that Tax does not need to be ubiquitinated to produce the effect observed on neuronal cells, since only a nonmodified 40-kDa form of Tax is secreted from MT2 cells. A retraction model with differentiated neuroblastoma cells (SH-SY5Y cells) gave us similar effects with culture media from both MT2 or PBMCs from HAM/TSP patients confirming the validity of using this cell line as well.^{19,23}

In conclusion, the interaction between secreted Tax and soluble SEMA-4D from HTLV-1-infected human lymphocytes led us to suggest a leading role for Tax in the SEMA-4D–Plexin 1B signaling pathway. The chemoattractant effect of sSEMA-4D on infected PBMCs exhibiting CRMP-2 pSer⁵²² implies a higher CNS infiltration of infected T cells, increasing both the occurrence of SEMA-4D T cells and sSEMA-4D levels in CSF. An insufficient number of reports on T cells makes it very interesting to continue to try to determine whether Tax and SEMA-4D are involved in

creasing the activities of Cdk5, GSK3 β (associated with Thr⁵⁰⁵ and Thr⁵¹⁴ phosphorylations), and/or Yes (to Tyr⁴⁷⁹ phosphorylation), some of them involved in cell migration.⁴¹ In this regard we have some studies in progress.

Further investigations will be necessary to evaluate the downstream effect of the Tax–sSEMA-4D complex to obtain a better understanding of its role in neuronal models. Further studies on HAM/TSP should consider the eventual role of Plexins or other semaphorins such as SEMA-3A, also secreted by lymphocytes and capable of producing alterations in neurite growth in neuronal cells.

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Author Disclosure Statement

No competing financial interests exist.

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