Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Letters 410 (2006) 94–99
Neuroscience Letters
www.elsevier.com/locate/neulet

Early dysregulation of cripto-1 and immunomodulatory genes in the cerebral cortex in a macaque model of neuroAIDS

Edward B. Stephens, Mollie Jackson, Lisa Cui, Eric Pacyniak, Rajani Choudhuri, Christopher S. Liverman, David S. Salomon, Nancy E.J. Berman *

University of Kansas Medical Center, Anatomy & Cell Biology, 3901 Rainbow Blvd., Kansas City, KS 66160, United States

Received 24 March 2006; received in revised form 15 June 2006; accepted 3 July 2006

Abstract

Human immunodeficiency virus type 1 (HIV-1) and related primate lentiviruses are known to enter the central nervous system (CNS) during the primary phase of infection. Neuroinvasion by simian immunodeficiency virus and simian human immunodeficiency virus (SHIV) is characterized by transient meningitis and astrocytosis. In this report, we used targeted cytokine cDNA arrays to analyze cortical brain tissue from four pig-tailed macaques inoculated for 2 weeks with pathogenic $SHIV_{50OLNV}$ and a normal age-matched pig-tailed macaque. Our results revealed that eight genes were significantly upregulated in all four macaques. These included: leukocyte interferon inducible peptide, corticotrophin releasing factor receptor 1, interleukin 6, CDW40 antigen, cysteine-rich fibroblast growth factor, neurotrophin 3, ciliary neurotrophin factor receptor and cripto-1. The upregulation of three of these genes was confirmed by reverse transcriptase PCR (RT-PCR). Since cripto-1 had not been previously identified within specific cell types within the primate central nervous system, we performed immunohistochemical studies, which revealed the presence of cripto-1 in neurons. RT-PCR studies demonstrated that cripto-1 mRNA was widely expressed in the CNS. These results indicate that immunomodulatory genes are upregulated during the primary phase of infection of the central nervous system. Cripto-1, which acts as a survival factor in tumor cells and may be neuroprotective, is expressed in neurons within the CNS and is upregulated during viral invasion.

Keywords: NeuroAIDS; Cripto; SHIV

Human immunodeficiency virus (HIV-1) invades the central nervous system (CNS) within weeks after infection and causes encephalitis (HIV-E) in approximately 25% of infected patients. Neurologic symptoms usually develop at a late stage of the disease when circulating $CD4^+$ T cells have dropped below 200 cells/μl [37]. The histopathology associated with HIV-E includes perivascular cuffing with lymphocytes and monocytes and formation of microglial nodules and giant cells [22]. Release of pro-inflammatory cytokines and chemokines from infected microglia/macrophages and astrocytes is thought to impair neuronal function directly and to disrupt the blood–brain barrier, exposing the brain to toxic molecules [1,40]. The possibility that expression of neuroprotective factors accompanies this increased cytokine expression has received less attention. Several non-human primate models have been used to study the neuropathogenesis of HIV-1. The SIV_{mac}/macaque model has provided useful information on the early events of neuroinvasion; both T cell tropic and neuropathogenic stains of SIV_{mac} enter the CNS early after inoculation, and development of SIV-E correlates with viral loads in the cerebrospinal fluid (CSF) [45]. A newer model uses the chimeric simian human immunodeficiency virus (SHIV) containing *tat*, *rev*, *vpu*, and *env* of HIV-1 in a genetic background of $SIV_{mac}239$. Pathogenic SHIVs are associated with high virus burdens, rapid loss of circulating $CD4^+$ T cells and depletion of T cell rich areas of the thymus, lymph nodes and spleen [18,34]. Macaques inoculated with pathogenic SHIVs generally succumb to their disease within 6–8 months, and develop neurological disease and neuropathology that is similar to SIV-E [19,20,27]. Previous studies have used microarray technology to examine altered gene expression after inoculation of rhesus macaques with SIVmac182 [29,30] or at end-stage disease after inoculation of rhesus macaques with pathogenic SHIV [7,39]. In the present study, we used targeted cDNA array technology to identify the host immunomodulatory genes that are upregulated in the cerebral cortical tissues during the early viremia phase of SHIV infection. Four macaques were examined

* Corresponding author. Tel.: +1 913 588 2712; fax: +1 913 588 2710.
E-mail address: nberman@kumc.edu (N.E.J. Berman).

0304-3940/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2006.07.066

2 weeks after inoculation with pathogenic $SHIV_{50OLNV}$. Our results demonstrate consistent upregulation of several genes, some of which may prove to be valuable prognostic markers for future development of SHIV-encephalitis.

Five macaques were used in this study: macaque AX62, an uninfected age-matched pig-tailed macaque and AX67, CM6G, CB4R, and CBRW [36], which were inoculated with pathogenic $SHIV_{50OLNV}$ 2 weeks prior to euthanasia [36]. All four these macaques developed astrocytosis, some developed a transient meningitis and disruptions in the blood–brain barrier, and all had extensive viral neuroinvasion [36,38]. Euthanasia procedures have been described previously [36]. The left halves of the brains were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer. Regions were blocked in a standard coronal plane into 6 mm blocks, cryoprotected in 30% sucrose in 0.1 M phosphate buffer, and frozen-sectioned at 50 μm using a sliding microtome. For routine histopathology, small blocks from the frontal, motor, parietal, occipital, and temporal cortex, corpus callosum, basal ganglia, thalamus, midbrain, pons, medulla, and cerebellum from the right half of the brains were fixed by immersion, and these blocks as well as blocks containing cervical, thoracic and lumbar spinal cord were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. In order to identify genes that were differentially expressed in SHIV infected and uninoculated macaques, cDNA array analysis was performed using the human cytokine cDNA array from Clontech (catalog #7744-1; Palo Alto, CA). The right half of the brain was dissected into blocks containing 14 different regions, and these blocks were frozen in liquid nitrogen and stored at −85 °C until used for RNA extractions. For microarray studies, RNA was prepared and analyzed as described previously [35]. Membranes were analyzed on a Packard Cyclone phosphoimaging system at a resolution of 50 μm, and spot intensities were measured using AtlasImage 2.0 software. We chose a 2.5-fold difference as the arbitrary cutoff value to allow for the animal-to-animal variation in gene expression that may occur in outbred macaques.

The results of cytokine cDNA array analysis were confirmed by performing RT-PCR for IL-6, leukocyte interferon inducible peptide (LIIF) and cripto-1 using separate RNA samples from parietal cortex. The oligonucleotides used in the RT-PCR amplification were based on human sequences in the GenBank: IL-6: 5′-CGCCTTCGGTCCAGTTGCCTTCT-3′ (sense) and 5′-ATCCAGATTCCAAGCATCCATC-3′ (antisense); LIIF: 5′-ATGCGCCAGAAGGCGGTATCCG-3′ (sense) and 5′-CTACTCCTCATCCTCCTCACTATC-3′ (antisense), and cripto-1: 5′-AAGCTATGGACTGCAGGAAGATGG-3′ (sense) and 5′-AGAAAGGCAGATGCCAACTAGC-3′ (antisense). The RT-PCR was performed with equal amounts of total RNA using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN) with an initial denaturation: step at 94 °C for 2 min followed by 10 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 68 °C for 45 s. This was followed by 25 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 68 °C for 2 min. At the end of the above cycling profile, a 10 min elongation step was performed at 68 °C. Following the PCR amplification, a 10 μl aliquot was removed and run on a 1.5% agarose gel and bands visualized by staining with ethidium bromide.

Table 1
Cytokine array genes upregulated ≥2.5-fold in all four macaques compared to uninfected, age-matched macaques

Gene or product	Average difference in expression (fold)	Accession number
Leukocyte interferon inducible peptide	30.09	X02492
Corticotropin releasing factor receptor 1 precursor (CRF-R; CRF1)	20.68	X72304
Interleukin-6 precursor	15.03	M14584
Cripto-1 (teratocarcinoma-derived growth factor)	9.56	M96955
CDW40 antigen	9.12	X60592
Cysteine-rich fibroblast growth factor	6.02	U64791
Neurotrophin-3 precursor	4.84	X52946
Ciliary neurotrophic factor receptor	4.74	M73238

For visualization of cripto-1 by immunohistochemistry, we used a rabbit polyclonal antibody (#1579) generated against a 17-mer peptide corresponding in sequence to the last 17 amino acids in the EGF-like domain of the human cripto-1 protein. This rabbit antibody recognizes full-length recombinant cripto-1 protein (∼28 kDa) by Western blotting and does not cross-react with any other EGF-like peptide in an ELISA such as EGF, TGF alpha, amphiregulin, HB-EGF or heregulin beta-1. The reactivity of this antibody is similar to the CR67 antibody previously described [26]. Frozen sections (50 μm) were incubated in this antibody (1:1000) overnight at room temperature, washed three times in PBS, and incubated in biotinylated goat anti-rabbit IgG (1:200) for 1 h. After ABC steps, sections were washed three times in PBS and rinsed in 0.5% Triton X-100 for 30 s and reacted with 0.5% diaminobenzidine with 0.1% H_2O_2. One set of controls for non-specific staining consisted of incubation of the sections with buffer in the place of the primary antibody. No staining was observed in these sections. A preabsorption control was performed by pre-incubating the rabbit polyclonal anti-cripto-1 antibody (1:1000 dilution) with the 17-mer peptide used to immunize the rabbits at a concentration of 50 μg/ml overnight at 4 °C prior to use in immunohistochemistry. The absence of staining after preabsorption confirmed that neuronal staining was specific for cripto-1.

In addition to the astrocyte activation reported previously [36], meningitis was observed at frontal (Fig. 1A), motor, parietal, occipital and temporal cortices and spinal cord (Fig. 1B). Occasional small microglial nodules were observed in the spinal cord (Fig. 1C). Results of microarray studies are provided in Table 1, which shows the genes that demonstrated expression at least 2.5-fold higher in all four inoculated macaques when compared to the uninfected control macaque (AX62). To confirm the upregulation of genes identified in the cDNA arrays, oligonucleotides based on the human sequences to cripto-1, leukocyte interferon inducible peptide, and IL-6 were used in RT-PCR assays of parietal cortex RNA samples. As shown in Fig. 2A, expression of all three genes was clearly upregulated in macaque