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Herpes simplex virus type-1 latency inhibits dendritic growth in sympathetic neurons

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Herpes simplex virus type-1 (HSV-1) initially infects mucoepithelial tissues of the orofacial region, the eye and to a lesser extent the genitalia. Subsequently, the virus is retrogradely transported through the axons of the sensory and sympathetic neurons to their nuclei, where the virus establishes a life-long latent infection. During this latency period, the viral genome is transcriptionally silent except for a single region encoding the latency-associated transcript (LAT). LAT has been shown to affect apoptosis, but little else is known regarding its effects on neurons. To understand how HSV-1 latency might affect dendrites in sympathetic neurons, we transfected primary cultures of sympathetic neurons obtained from rat embryos, with LAT expressing plasmids. LAT inhibited initial dendritic growth and induced dendritic retraction in sympathetic neurons. Latent HSV-1 infection of cultured sympathetic neurons inhibited dendritic growth indicating that this is likely also a consequence of natural infection.

Keywords: Herpes simplex virus type-1; Latency; LAT; Sympathetic neurons; Bone morphogenetic protein-7; Dendrites

Introduction

Herpes simplex virus type-1 (HSV-1) is a member of the alpha-herpes virus subfamily and infects two thirds of the United States population (Schillinger et al., 2004). HSV-1 initially infects the mucoepithelial tissues of the orofacial region and, to a much lesser extent, of the genitalia. Subsequently, the virus is retrogradely transported to the cell bodies of the sensory and sympathetic neurons in the corresponding ganglia (Bastian et al., 1972; Warren et al., 1978). During latent neural infection with HSV-1, significant gene expression is restricted to one mRNA, the latency associated transcript (LAT) (Stevens et al., 1987; Deatly et al., 1987). The primary transcript of the HSV-1 LAT gene is 8.3 kb long. However, this is rapidly processed to yield shorter transcripts, the most prominent of which is a stable 2 kb intron (Farrell et al., 1991). This transcript has been observed exclusively in the nuclei of the latently infected neurons (Wagner et al., 1988).

LAT has been shown to regulate the establishment of viral latency and reactivation (Block et al., 1993; Thompson and Sawtell, 1997) and inhibits apoptosis in sensory neurons (Perng et al., 2000). In our studies, we transfected plasmids expressing the 2 kb LAT intron into primary rat sympathetic neurons to examine the effects of LAT on dendritic growth. We show here for the first time that HSV-1 latency inhibits the growth of dendrites, thus indicating a novel role for LAT in the biogenesis of these processes. Recently, it has been shown that LAT encodes a microRNA that interferes with the TGF-β signaling pathway (Gupta et al., 2006). We show evidence that LAT affects dendritic growth through inhibition of the signaling pathway of the bone morphogenetic protein-7 (BMP-7), which is a member of the TGF-β family.

Materials and methods

Materials

Human recombinant bone morphogenetic protein-7 (BMP7) was generously provided by Curis (Cambridge, MA). Acyclovir was purchased from Sigma (St. Louis, MO).

Plasmids

A plasmid coding enhanced green fluorescence protein (pEGFP-C1) was purchased from Clontech (Clontech, Palo Alto, CA). The plasmid expressing the VZVIE63 protein has been previously described (Zuranski et al., 2005). Plasmids expressing the full-length 2.0 kb LAT intron as a lariat and truncations were a generous gift from Dr. Lawrence Feldman (UCLA, LA, CA).

Cell cultures

Superior cervical ganglia were dissected from embryos (E20) of Holtzman rats (Harlan Sprague–Dawley, Rockford, IL). The

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cells were dissociated after treatment with trypsin (2.5 mg/ml) and collagenase (1 mg/ml) for 35 min. Cells were plated on poly-D-lysine-coated (200 μg/ml) coverslips and maintained on serum-free medium containing β-nerve growth factor (NGF: 100 ng/ml). One day later, the cultures were treated with medium containing the antimitotic drug cytosine-β-D-arabinofuranoside (ara-c) (1 μM) for 2 days to eliminate non-neuronal cells, and then the cultures were allowed to recover for 2 days before transfection.

Transfection

Cells were cotransfected with a plasmid encoding the enhanced green fluorescence protein (pEGFP-C1, Clontech, Palo Alto, CA) and the expression vector LTF900 containing the LAT 2.2 kb insert, or one of a set of four expression vectors (LTF901, LTF902, LTF903 and LTF904) containing different deletion mutants of LAT. In control experiments, cells were cotransfected with pEGFP-C1 and the pCDNA3.1 vector (Invitrogen, CA). Transfection was performed using Lipofectamine 2000 (Invitrogen, CA). Briefly, cells were treated with 150 μl of DMEM containing 1.6 μg of plasmid DNA and 6 μg of Lipofectamine for 6 h. Subsequently, cells were washed and allowed to recover for 2 days prior to treatment with BMP7.

In situ hybridization

Antisense and sense riboprobes for LAT were prepared as follows: a fragment of LAT (814 basepairs) was amplified by PCR, and then subcloned into the PCR II-TOPO vector (Invitrogen, Carlsbad, CA). Digoxigenin-labeled riboprobes were generated by in vitro transcription according to the manufacturer's instructions (Roche, Indianapolis, ID). Cultures were fixed with 4% formaldehyde for 1 h and washed twice with 2× SSC. Cultures were treated with proteinase K (5 μg/ml) for 10 min then washed twice with 2× SSC. Cells were incubated at 50°C for 3 h with a pre-hybridization solution which contained 50% formamide, 4× SSC, 1 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, yeast tRNA (250 μg/ml) and denatured salmon sperm DNA (250 μg/ml).

Cells were hybridized by incubation at 50°C for 16 h with 2 μg/ml of probe in hybridization solution. The LAT signal was detected using anti-digoxigenin Fab fragment conjugated to rhodamine (Roche, Indianapolis, ID) and visualized by fluorescence microscopy.

Morphological analysis

Immunocytochemistry was used to analyze cellular morphology as previously described (Bruckenstein and Higgins, 1988). Briefly, Sympathetic neurons were fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.1% Triton X-100 for 4 min, prior to incubation with 5% bovine serum albumin for 2 h at room temperature. Cells were reacted with monoclonal antibodies to microtubule-associated protein-2 (MAP2) (Sternberger Immunocytochemicals, Baltimore, MD) overnight, and then with anti-mouse rhodamine conjugated secondary antibodies (Roche, Indinapolis, IN). Photographs of neurons were taken and total lengths of dendrites were measured with SPOT software (Diagnostic instruments, Sterling Heights, MI). Rabbit polyclonal phospho-Smad antibodies (Cell Signaling Technology, Danvers, MA) were used for immunostaining of phospho-smad in sympathetic neurons, with the use of anti-rabbit rhodamine-conjugated secondary antibodies (Roche, Indinapolis, IN).

Propagation of virus

Stocks of HSV-1 strain F were generated via infection of Vero or MeWo cell monolayers as previously described (Ruyechan and Weir, 1984). Stocks were titrated using standard protocols (Ruyechan et al., 1979) and stored at −80° prior to use.

Latent HSV-1 infection of neuronal cultures

Cultures of sympathetic neurons from the superior cervical ganglia were treated with ara-c (1 μM) for 4 days to eliminate non-neuronal cells. Cells were allowed to recover for 2 days, and then they were treated with acyclovir (50 μM) for 24 h. Subsequently, cultures were infected with HSV-1 (F strain) (MOI 1) in the presence of acyclovir (50 μM) and bone morphogenetic protein-7 (BMP7) (30 ng/ml). Three days later, the medium was replaced with acyclovir-free C2 medium which contained BMP7 (30 ng/ml), and 5 days later the cultures were fixed with 4% paraformaldehyde.

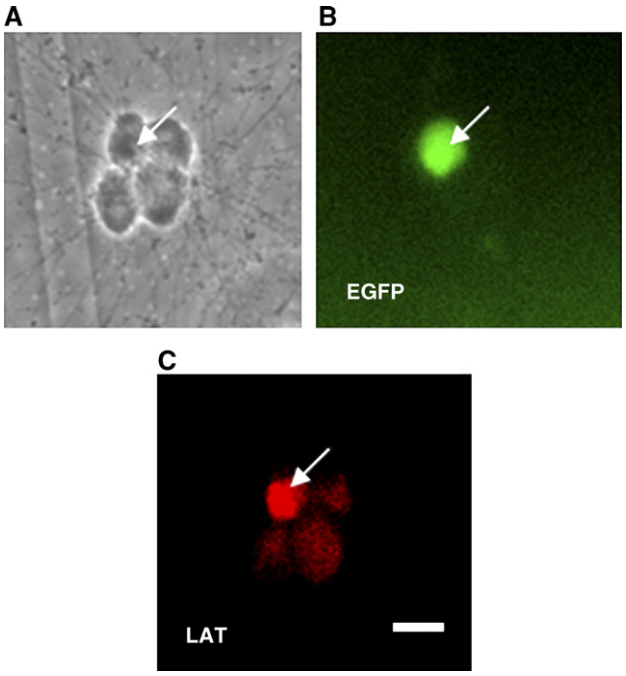

Fig. 1. Cotransfection of EGFP and LAT plasmids. Sympathetic neurons were cultured on gridded coverslips. After elimination of non-neuronal cells, neurons were cotransfected with plasmids containing EGFP and LAT. Two days later, EGFP-expressing neurons were photographed and mapped, and then in situ hybridization was done for detection of LAT. The EGFP-expressing cells were relocated and identified, and the LAT-positive neurons were detected. Approximately 89% of the EGFP-expressing neurons were LAT-positive ($n=35$). (A) Phase-contrast micrograph of a group of four sympathetic neurons. (B) A green fluorescence micrograph showing one of the four neurons expressing EGFP. (C) Red fluorescence micrograph of the same group of neurons after in situ hybridization, showing that the EGFP-expressing neuron is also expressing LAT, whereas none of the other neurons was LAT-positive. The white arrows indicate the position of the nucleus. Scale bar is 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)