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Original article

Expression and role of the TGF-β family in glial cells infected with Borna disease virus

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Abstract

A previous study revealed that the expression of the Borna disease virus (BDV)-encoding phosphoprotein in glial cells was sufficient to induce neurobehavioral abnormalities resembling Borna disease. To evaluate the involvement of the TGF-β family in BDV-induced changes in cell responses by C6 glial cells, we examined the expression levels of the TGF-β family and effects of inhibiting the TGF-β family pathway in BDV-infected C6 (C6BV) cells. The expression of activin βA and BMP7 was markedly increased in BDV-infected cells. Expression of Smad7, a TGF-β family-inducible gene, was increased by BDV infection, and the expression was decreased by treatment with A-83-01 or LDN-193189, inhibitors of the TGF-β/activin or BMP pathway, respectively. These results suggest autocrine effects of activin A and BMP7 in C6BV cells. IGFBP-3 expression was also induced by BDV infection; it was below the detection limit in C6 cells. The expression level of IGFBP-3 was decreased by LDN-193189 in C6BV cells, suggesting that endogenous BMP activity is responsible for IGFBP-3 gene induction. Our results reveal the regulatory expression of genes related to the TGF-β family, and the role of the enhanced BMP pathway in modulating cell responses in BDV-infected glial cells.
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Keywords: Borna disease virus; TGF-β family; Glial cells; BMP; IGFBP-3

1. Introduction

Borna disease virus (BDV) is a non-segmented negative-strand RNA virus belonging to the family *Bornaviridae* [1]. The genome of BDV encodes at least 6 proteins: a nucleoprotein (N), nonstructural protein (X), phosphoprotein (P), matrix protein (M), envelope protein (G), and RNA polymerase (L) [2]. BDV naturally infects a wide range of warm-blooded hosts [3]; BDV infection induces severe signs of neurological disease, including behavioral abnormalities [3]. Responses to BDV infection vary according to differences in the species, animal strain, age of the host at the time of infection, or viral strain, indicating that host-dependent factors as well as virus-specific factors determine the onset of Borna disease [4–6]. However, the precise mechanism underlying the BDV-induced onset of behavioral disorders currently remains unclear.

Previous studies have attempted to identify the BDV-derived molecule(s) responsible for the onset of Borna disease in BDV-infected animals [7,8], and found that the forced expression of P in glial cells [7] but not N in neurons and glial cells [8] induced behavioral abnormalities such as enhanced intermale aggressiveness, hyperactivity, and spatial reference memory deficits resembling the neurobehavioral abnormalities exhibited in BDV-infected animals. Furthermore, the ectopic expression of BDV P did not affect neurodegenerative reactions [7]. Thus, functional modulations in glial cells have been implicated in these behavioral abnormalities [7].

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We previously demonstrated that the expression of transforming growth factor (TGF)-β family members and their signal components was altered in the brains of 3-week-old rats infected with BDV [6]; the expression of TGF-β1, activin βE, and GDF15 was increased in the brain in response to BDV infection, whereas that of TGF-β2, inhibin α, and BMP2 was decreased [6]. Furthermore, the expression of TβRII and ALK5, receptors for TGF-β [9,10], was shown to be increased in BDV-infected brains, while that of Smads, signal mediators and regulators of the TGF-β family [9,10], was generally increased by BDV infection [6]. Cell growth and differentiation are potently regulated by the TGF-β family in many types of cell, and alteration of TGF-β family signal is implicated in the pathogenesis of diseases through disturbances of cell response [9,10]. In view of the (patho-)physiological role of the TGF-β family, we hypothesized the involvement of the TGF-β family in the BDV-induced functional modulation of the central nervous system; previous findings on the Borna disease-like phenotype resulting from the glial expression of BDV P [7] and modulated expression of TGF-β family signaling components [6] prompted us to hypothesize that BDV infection in glial cells induces behavioral disturbances through the modulation of TGF-β family signaling. In order to validate our hypothesis, the present study examined 1) the regulatory expression of the signal components of the TGF-β family in glial cells infected with BDV, and 2) the effects of inhibiting the TGF-β family pathway on cell responses in BDV-infected glial cells.

2. Materials and methods

2.1. Materials

The following reagents were purchased: TGF-β1 and BMP7 were from R & D Systems (Minneapolis, MN, USA); LDN-193189, an inhibitor of BMP type I receptors [11,12], was from Stemgent (San Diego, CA, USA); A-83-01, an inhibitor of TGF-β/activin type I receptors [13], was from Calbiochem (La Jolla, CA, USA); rabbit polyclonal antibodies against phospho-Smad1 (Ser463/Ser465)/Smad5 (Ser463/Ser465)/Smad8 (Ser465/Ser467) (#9511), phospho-Smad2 (Ser465/Ser467) (#3101), or phospho-ERK (Thr202/Tyr204) (#9101) were from Cell Signaling Technology (Danvers, MA, USA); and a mouse monoclonal antibody against α-tubulin (B-5-1-2) was from Abcam (Cambridge, MA, USA).

2.2. Cell culture

C6 glioma cells [14] and C6 glioma cells infected with the BDV He/80 strain (C6BV, [15]) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. In order to examine the effects of inhibitors of the TGF-β family on gene expression, C6 and C6BV cells were plated at the density of 1×10^4/mL. Sixteen hours after plating, cells were treated with A-83-01 (5 μM) or LDN-193189 (100 pM) or both for 72 h.

2.3. Western blot

C6 and C6BV cells were plated at the density of 2.5×10^5/mL to examine the phosphorylation of Smad and ERK. Twenty hours after plating, cells were cultured in DMEM medium with 0.2% FBS for 4 h to eliminate the effects of serum on phosphorylation. Cells were pretreated with A-83-01 (5 μM) or LDN-193189 (100 pM) for 15 min, followed by a treatment with TGF-β1 (100 pM) or BMP7 (2 nM) for 1 h. Western blot analyses were performed as described previously [16]. Immunoreactive proteins were visualized using the ECL Select Western blotting detection system (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's protocol.

2.4. RNA isolation, cDNA synthesis, and real-time quantitative PCR

Total RNA was isolated from C6 and C6BV cells using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). The concentration of RNA was calculated from absorbance at 260 nm. The ratio of absorbance at 260 nm to that at 280 nm was comparable among samples. The cDNA was synthesized from 1 μg of total RNA by the ReverTra Ace qPCR Master Mix (Toyobo, Osaka, Japan), according to the manufacturers' protocols. The cDNA corresponding to 5 ng of total RNA, i.e., 0.5% of the cDNA, was used as a template to evaluate expression levels, except for IGFBP-3, for RT-quantitative PCR (RT-qPCR); the cDNA corresponding to 20 ng of total RNA (2% of the cDNA) was used to examine the expression of IGFBP-3. qPCR was performed using Thunderbird qPCR Mix (Toyobo) in Applide Biosystems StepOnePlus Real Time PCR Systems (Life Technologies, Carlsbad, CA, USA), according to the manufacturers' protocol. The qPCR profile was as follows: after denaturing for 20 s at 95 °C, 40 cycles consisted of 15 s at 95 °C and 60 s at 60 °C. The oligonucleotide primers for RT-qPCR and PCR efficiency [17] are shown in Table 1. All the primer sets were designed to span an exon–exon junction to exclude the possibility of amplification of genomic DNA. After 40 cycles of RT-qPCR, the dissociation (melting) curve of the products was examined by changes in the ramp temperature from 60 °C to 95 °C. Each sample showed a single peak, suggesting the expected PCR products. The abundance of gene transcripts was calculated by the standard curve method, and the value was normalized against HPRT1, GAPDH or TBP. The expression level of genes in C6 cells treated without the inhibitor was set to 1, except for IGFBP-3. Since the expression of IGFBP-3 in C6 cells was below the detection limit, its level in C6BV cells treated without the inhibitor was set at 1. The cell culture experiments, i.e., comparison of gene expression between C6 cells and C6BV cells as well as effect of inhibitors for the TGF-β family pathway on gene expression, were performed at least two times, and showed similar results.

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