Molecular and Cellular Neuroscience 70 (2016) 76–85

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Vitronectin promotes the progress of the initial differentiation stage in cerebellar granule cells

Kei Hashimoto, Fumi Sakane, Natsumi Ikeda, Ayumi Akiyama, Miyaka Sugahara, Yasunori Miyamoto *

Graduate School of Humanities and Sciences, Ochanomizu University, Otsuka 2-1-1, Bunkyo-ku, Tokyo 112-8610, Japan

ARTICLE INFO

Article history:
Received 17 June 2015
Revised 16 November 2015
Accepted 27 November 2015
Available online 27 November 2015

Keywords:
Vitronectin
Development of the cerebellum
Cerebellar granule cell
Differentiation
Migration

ABSTRACT

Vitronectin (VN), which is an extracellular matrix protein, is known to be involved in the proliferation and differentiation of primary cultured cerebellar granule cell precursors (CGCPs); however, the effect of VN is not fully understood. In this study, we analyzed the effects of VN loss on the proliferation and differentiation of CGCPs in VN knockout (VNKO) mice *in vivo*. First, immunohistochemistry showed that VN was distributed in the region from the inner external granule layer (iEGL) through the internal granule layer (IGL) in wild-type (WT) mice. Next, we observed the formation of the cerebellar cortex using sagittal sections of VNKO mice at postnatal days (P) 5, 8 and 11. Loss of VN suppressed the ratio of NeuN, a neuronal differentiation marker, to positive cerebellar granule cells (CGCs) in the external granule layer (EGL) and the ratio of CGCs in the IGL at P8, indicating that the loss of VN suppresses the differentiation into CGCs. However, the loss of VN did not significantly affect the proliferation of CGCPs. Next, the effect of VN loss on the initial differentiation stage of CGCPs was examined. The loss of VN increased the expression levels of Transient axonal glycoprotein 1 (TAG1), a marker of neurons in the initial differentiation stage, in the cerebella of VNKO mice at P5 and 8 and increased the ratio of TAG1-positive cells in the primary culture of VNKO-derived CGCPs, indicating that the loss of VN accumulates the CGCPs in the initial differentiation stage. Taken together, these results demonstrate that VN promotes the progress of the initial differentiation stage of CGCPs.

1. Introduction

Cerebellar granule cells (CGCs) constitute the vast majority of neurons in the cerebellum. Therefore, determining the mechanisms underlying the proliferation of cerebellar granule cell precursors (CGCPs) and the differentiation of CGCPs into CGCs is important for understanding cerebellar development. The developing cerebellar cortex is formed from four layers: the external granule layer (EGL), the molecular layer (ML), the Purkinje cell layer (PcL), and the internal granule layer (IGL). The proliferation of CGCPs is active in the outer external granule layer (oEGL) (Miale and Sidman, 1961). After this proliferation stage, the CGCPs stop the cell cycle in the inner external granule layer (iEGL), initiate differentiation into CGCs, and migrate through the layers to form the iEGL, the ML, and the PcL. Additionally, the parallel fibers of CGCPs are elongating and the CGCPs are differentiating into CGCs (Miale and Sidman, 1961; Rakic, 1971; Hatten, 1999). Differentiation into CGCs is completed when the cell bodies of CGCs reach the IGL. Proliferation of CGCPs occurs actively at postnatal days (P) 5–8 and subsequently ends at approximately P15 in mice (Hatten et al., 1997). Additionally, the differentiation of CGCPs into CGCs is completed in the IGL at approximately P20 (Hatten et al., 1997).

Extracellular matrix proteins, such as laminin (LN), CCN3/NOV, and heparan sulfate proteoglycan, regulate the proliferation, differentiation and migration of CGCs (Hatten, 1999). During cerebellar development, LN is expressed in the oEGL, ML and PcL and promotes the proliferation of CGCPs through its receptor β1 integrin (Pons et al., 2001). CCN3/NOV, a member of the CCN family, reduces Sonic hedgehog (Shh)- induced proliferation of CGCPs by stimulating GSK3β through αvβ3 integrin (Le Dreau et al., 2009). Additionally, heparan sulfate proteoglycan promotes the proliferation of CGCPs through an interaction between the heparan sulfate chains and Shh (Rubin et al., 2002).

Vitronectin (VN) is a cell adhesion protein that can be detected in plasma and the extracellular matrix. It is known that VN acts as a scaffold during cell anchoring and is involved in the control of blood coagulation, fibrinolysis, complement activity, and cell migration (Schvartz et al., 1999). Furthermore, it was revealed that VN regulates the proliferation and differentiation of various types of neuron precursors during

Abbreviations: VN, vitronectin; CGC, cerebellar granule cell; CGCP, cerebellar granule cell precursor; WT, wild- type; VNKO, VN knockout; iEGL, inner external granule layer; IGL, internal granule layer; P, postnatal day; EGL, external granule layer; TAG1, transient axonal glycoprotein 1; ML, molecular layer; PcL, Purkinje cell layer; oEGL, outer external granule layer; LN, laminin; Shh, Sonic hedgehog; CREB, cyclic-AMP responsive element binding protein; PBS, phosphate-buffered saline; BrdU, bromodeoxyuridine; PFA, paraformaldehyde; PHH3, phospho-histone H3; DIG, digoxigenin; DEPC, diethylpyrocarbonate; GFAP, glial fibrillary acidic protein; DAPI, 4′, 6-diamidino-2-phenylindole dihydrochloride.

* Corresponding author.
E-mail address: miyamoto.yasunori@ocha.ac.jp (Y. Miyamoto).

http://dx.doi.org/10.1016/j.mcn.2015.11.013
1044-7431/

development. For example, VN is detected in the developing chick retina and sustains both proliferation and differentiation of cultured neuroepithelial cells from embryonic day 5 retinas (Martinez-Morales et al., 1995). Additionally, VN expression is increased with the generation of motor neuron differentiation in the floor plate of the embryonic chick neural tube, and anti-VN antibodies reduce the number of motor neurons (Martinez-Morales et al., 1997; Pons and Marti, 2000).

VN is also involved in the development of CGCPs during cerebellar formation. VN suppresses Shh-induced proliferation and promotes the differentiation of CGCPs in primary cultures (Murase and Hayashi, 1998; Pons et al., 2001; Katic et al., 2014). Further analyses have suggested that the suppression of CGCP proliferation is regulated by VN-induced phosphorylation of the cyclic-AMP responsive element binding protein (CREB) in primary cultures of CGCPs from rats (Pons et al., 2001). Moreover, blocking VN binding to CGCPs inhibits the centrosome positioning at the base of the axons in primary cultures of CGCPs from mice (Gupta et al., 2010). This centrosome positioning dictates the orientation of axon elongation. Therefore, VN plays an important role in the regulation of the proliferation and differentiation of cultured CGCPs and in the orientation of axon elongation of CGCs in culture. However, the roles of VN in the developing CGCPs have not been understood fully in the developing cerebellum.

In this study, we analyzed the effects of VN on the proliferation and the differentiation of CGCPs during cerebellar development using VN-knockout (KO) mice. In the cerebellar cortex of mice at P8, the loss of VN reduced the number of differentiated CGCs but did not significantly affect the proliferation of CGCPs. Additionally, the loss of VN increased the expression level of Transient axonal glycoprotein 1 (TAG1), which is a marker of neurons in the initial differentiation stage, at P5 and 8. The loss of VN also increased the ratio of CGCPs in the initial differentiation stage in primary cultures. These findings demonstrate that VN plays an important role in the progress of the initial differentiation stage of CGCPs.

2. Material and methods

2.1. Animals

The C57/BL6J mice were obtained from the Charles River lab in Japan (Yokohama, Japan), and the VNKO mice were generously provided by Dr. David Ginsburg (University of Michigan). All experiments were approved by the Institutional Animal Care and Use Committee of Ochanomizu University, Japan (animal study protocols 12003, 13003, and 14005) and followed the guidelines established by the Ministry of Education, Science and Culture in Japan. The C57/BL6J and VNKO mice were kept on a 12 h light–12 h dark cycle at 22 °C.

2.2. Western blotting

Mouse cerebella at P2, 5, 8, 11, 14 and 20 were collected in phosphate-buffered saline (PBS) and lysed in sample buffer (25 mM Tris–HCl, 5% glycerol, 1% SDS, 0.05% bromophenol blue, 1% 2-mercaptoethanol). The supernatants of these sample solutions were separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes (ATTO, Tokyo, Japan). The membranes, which were blocked with 0.3% skim milk overnight, were incubated with primary antibodies for 1 h and horseradish peroxidase secondary antibodies for 1 h. The primary antibodies in this study included: anti-VN (1:5000; a gift from Dr. Masao Hayashi, Ochanomizu University, Tokyo, Japan), anti-β-actin (1:10,000; G043; Applied Biological Materials, Richmond, BC) and anti-TAG1 (1:1000; C9C5; Cell signaling Technology, Beverly, MA). The bands were visualized with an enhanced luminescent reagent (ATTO) and quantified using LAS3000 and Multi Gauge-ver. 2.2 (GE Healthcare UK Ltd., Little Chalfont, England).

2.3. Immunohistochemistry

Immunohistochemistry was performed using modifications of the procedure described by Sakane and Miyamoto (Sakane and Miyamoto, 2013). Newborn mice were injected intraperitoneally with 50 μg/g bromodeoxyuridine (BrdU) (BD Biosciences, San Jose, CA) at P3, 6 and 9 and sacrificed 48 h later. Mice at P5, 8 and 11 were perfused and fixed with 4% paraformaldehyde (PFA), and the brains were fixed with 4% PFA overnight. Serial sagittal sections (20 μm thick) through the cerebella were obtained using a cryostat (Leica CM1850; Leica Microsystems, Wetzlar, Germany). The sections were incubated with primary antibodies overnight and secondary antibodies for 1 h. The primary antibodies in this study included: anti-VN (1:500; LSL-LB-2096; Cosmo Bio, San Diego, CA), anti-Calbindin (1:3000; D28K; Sigma-Aldrich, St. Louis, MO), anti-BrdU (1:500; MAB3222; Merck Millipore, Birellica, MA), anti-Ki67 (1:200; RM9106-S0; Thermo Fisher Scientific, San Jose, CA), anti-phospho-histone H3 (PHH3) (1:200; 06–570; Merck Millipore), anti-NeuN (1:400; MABN140, Merck Millipore), anti-TAG1 (1:50; 4D7; Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-active Caspase3 (1:200; AF835; R&D systems, Minneapolis, MN). Confocal images with a single plane in the immunostained samples were captured at magnification 40× with a confocal microscope (LSM710, Carl Zeiss, Germany).

2.4. In situ hybridization

In situ hybridizations were performed as described previously (Sakane and Miyamoto, 2013). The RNA antisense probes were prepared using plasmid cDNA clones for VN transcribed with SP6 polymerase using digoxigenin (DIG)- labeling reagents and a DIG RNA labeling kit (Roche Diagnostic Corporation, Indianapolis, IN). The brains were fixed overnight at room temperature in 4% PFA in diethylpyrocarbonate (DEPC)-treated PBS, cryoprotected in 15 and 30% sucrose in DEPC-treated PBS, and embedded in OCT compound. The frozen brains were processed into 20 μm sections with a cryostat. The sections were prehybridized with hybridization buffer (Amresco, Solon, OH) for 2 h before hybridization buffer containing DIG-labeled riboprobes (200–400 ng/ml) was applied at 65 °C overnight. To visualize the results, we used an alkali phosphatase-labeled DIG antibody and BM purple (Roche). The images were captured using a microscope (FSX100, Olympus, Japan).

2.5. Granule cell culture

Primary culture of CGCPs was performed using a modified version of the procedure described by Weber and Schachner (Weber and Schachner, 1984). P6 mouse cerebella were aseptically isolated and the meninges were removed. The cerebella were dissociated with 1% trypsin and DNase I (Roche Diagnostics) in Hanks balanced salt solution (135.8 mM NaCl, 5.36 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1 mg/ml glucose, $NaHCO_3$) for 13 min at room temperature. The cells were dispersed by pipetting and passed through a nylon net with a mesh size of 70 μm. The dissociated cells were suspended in serum-free Neurobasal Medium (Life Technologies, Carlsbad, CA) with 500 μM L-glutamine, 2% B-27 Supplement (Life Technologies), 25 mM KCl, 50 unit/ml penicillin, 50 μl/ml streptomycin and 10 nM smoothened agonist (a gift from Curis Inc., Lexington, MA). The cell density was quantified and adjusted to 6.0×10^5 cells/ml. Next, the suspensions were plated on poly-L-lysine-coated cover slips in 24-well plates and cultured in 5% CO_2 at 37 °C for 72 h. At 24 h after plating, all culture media were replaced with fresh media. To label the cells with BrdU, 20 μM BrdU was added to the culture media at 24 h prior to fixation with 4% PFA. When we added VN, we added 2.5 μg/ml or 5.0 μg/ml VN to all culture media.

Immunofluorescence staining using the primary cultures was performed with primary antibodies overnight and secondary antibodies for 1 h. The primary antibodies in this study included: anti-glial fibrillary acidic protein (GFAP) (1:200; G9269; Sigma-Aldrich) as well as