쥐의 신경세포 배양에 의한 수초 발생과 sindbis 바이러스 감염에 의한 수초 억제

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Generation of myelination with neural cell cultures in rats and suppression of myelination by infection of sindbis virus

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요 약

본 연구는 뉴런 세포와 슈반 세포의 공동 배양에 의한 수초화 발생 과정과 herpes simplex virus-1 감염에 의한 탈수초화 발생과정을 전자 현미경과 분자생물학적 분석에 의하여 확인하고자 하였다. 쥐의 배아로부터 후근신경절(dorsal root ganglion, DRG)을 분리하여 슈반(Schwann) 세포와 뉴런 세포(neuronal cell)를 *in vitro*에서 각각 배양하였다. 유사 분열 억제인자로 처리한 뉴런세포와 정제된 슈반 세포를 함께 공동 배양을 하여 수초화를 발생시켰다. 이렇게 수초화된 공동 배양 세포에 herpes simplex virus-1를 감염시켜 탈수초화를 진행시켰다. 수초 형성의 존재를 의미하는 myelin protein zero(MPZ) 항체를 사용하고 전자 현미경을 이용하여 수초 발생 및 탈수초화 과정을 관찰하였다. 이 연구는 과학 기술부, ICT 및 미래 계획 (NRF-2016R1A2B4016552 및 2017R1A2B3005753)이 자금을 지원하는 국립연구 재단 (NRF)을 통한 기초 연구 프로그램의 지원을 받았다.

ABSTRACT

The dorsal root ganglion (DRG) was isolated from mouse embryos and Schwann cells and neuronal cells were cultured *in vitro*. The neurons and Schwann cells were cultured separately and the two kinds of cells were cultured together for three weeks. Generation of myelination was confirmed by transmission electron microscope and confocal microscope using a myelinaion protein, myelin protein zero (MPZ) antibody. The sindbis virus was infected for three days in the myelinated culture cells and then demyelination was carried out. The process of demyelination was also confirmed by transmission electron microscopy and confocal microscopy using myelin protein zero (MPZ) antibody. The study was supported by a Basic Research Program through the National Research Foundation (NRF) funded by the Ministry of Science and Technology, ICT and Future Plans (NRF-2016R1A2B4016552 and 2017R1A2B3005753).

키워드

수초, 탈수초, myelin protein zero 항체, sindbis virus

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Ⅰ. Introduction

A demyelinating disease is any disease of the nervous system in which the myelin sheath of neurons is damaged. This impairs the conduction of signals in the affected nerves, causing impairment in sensation, movement, cognition, or other functions depending on which nerves are involved. Demyelinating diseases are traditionally classified in two kinds: demyelinating myelinoclastic diseases and demyelinating leukodystrophic diseases. In the first group, a normal and healthy myelin is destroyed by a toxic, chemical, or autoimmune substance. In the second group, myelin is abnormal and degenerates [1].

Diseases carrying demyelination process include diverse immunopathologic syndromes in which myelin, the fatty covering of nerve cell fibers in the brain, optic nerve and spinal cord is destroyed. Even though the pathogenesis of demyelination are unknown, one hypothesis is that autoimmunity to antigens of the central nervous system (CNS) is triggered by environmental factors, such as viral infections, in genetically susceptible individuals, and that the activated immune response leads to myelin destruction. Among them, the major diseases associated with degradation of the myelin sheath is multiple sclerosis [2].

Researchers developed a DRG neuron and schuwann cells co-culture system for myelin research *in vitro* [3]. The study of myelination has been facilitated by the availability to isolate and establish pure population of Schwann cells and Neuronal cells from primary Schwann cells. Mammalian DRG neuron cells can survive and regenerate in culture [4, 5, 6].

Some viruses such as Theiler's virus, mouse hepatitis virus (MHV), corona, measles, SINV, and Herpes simplex virus-1 are known as cause of inducing demyelination in nervous system of mice or rats. Virus like Herpes simplex virus-1 infection induces a demyelinating encephalomyelitis in the central nervous system of mice. Mice and rats are used as an important model for the study of myelination and demyelination research *in vitro* and *in vivo*.

In vitro myelination had been established by co-culturing with pure populations of primary Schwann cells and primary neuronal cells. These pure populations of primary Schwann cells and primary neuronal cells were driven from DRG of rat embryos. This method produced highly purified populations of Schwann cells and neuronal cells faster than any other conventional method.

In this study, The neurons and Schwann cells were cultured separately and the two kinds of cells were cultured together for three weeks. Generation of myelination was confirmed by transmission electron microscope and confocal microscope using a myelinaion protein, myelin protein zero (MPZ) antibody. The sindbis virus was infected for three days in the myelinated culture cells and then demyelination was carried out. The process of demyelination was also confirmed by transmission electron microscopy and confocal microscopy using myelin protein zero (MPZ) antibody.

II. Materials and methods

A. preparation of embryonic DRG

Embryos of mouse were decapitated with spring scissors and transfered the head to a pre-labeled 1.5 mL Eppendorf tube prepared. The tips of spring scissors vertically were inserted into the rostral end, just anterior to the vertebral column and begin making 3−4 sequential cuts from rostral to caudal (from head to tail) direction. The ganglia from the dorsal root starting from one end of the spinal cord were pinched off. All the ganglia (approximately 37−42 ganglia/spinal cord/embryo) were removed and repeated with the step for all the spinal cords. The 1.5 mL of 0.25% trypsin were added to each dish and placed the dishes into a 37℃ air incubator for 15−20 min. It were added with 10 mL of L-15/10% FBS into each tube and centrifuged the ganglia at 50 *g* for 5 min. The pellet was added and resuspended with 10 mL of L-15/10% FBS. After centrifugation for 5 min as above, it was added with 1 mL of DRG plating medium to each tube. Using narrow-bored glass Pasteur pipettes by flaming the tips, the pellet was triturated 10−15 times and added with additional 1 mL of DRG plating medium to the cell suspension. This dissociated cells in an uncoated 35 mm dish were incubated.

B. suspension of the embryonic dorsal root ganglion cells

After 18 hr, plating of the dissociated DRG was replaced the medium with 2 mL of N2/NGF. Under the serum-free condition, fibroblast growth is suppressed while axon-associated Schwann cell precursors continue to proliferate. There is no need

to change the medium. After 6-7 days, Schwann cell-neuron network forming axon bundles was transferred into culture medium.

C. purification of Schwann cells.

After collecting Schwann cell−neuron networks, the cells were pelleted by centrifugation at 200 *g* for 5 min.

The pellet was resuspend in trypsin-collagenase solution and incubated at 37 ℃ for 30 min. The reaction was stopped by adding Schwann cell medium and the cells were pelleted as above. The pellet was resupended in 1 ml of anti-Thy 1.2 antibody solution and incubated 30 min at 37 ℃. After centrifugation, pellet was resupended in 1 ml of rabbit complement solution and incubated 30 min at 37℃. Cells were plated onto a poly-L-lysine coated 100 mm culture dish in Schwann cell medium and incubated 30 min at 37℃. Next day, bipolar, spindle-shaped Schwann cells were changed the medium to N2-Schwann cell growth medium. Neuregulin and Forskolin contained in the growth medium start to stimulate Schwann cell proliferation while growth of any contaminating fibroblasts is suppressed under the serum-free condition. Neurons should die off within 2 days in the absence of NGF in the medium [7]. After 6-7 days, the cells were replated onto new poly-L-lysine-coated plates at a density of 1×10^6 cells/100 mm plate.

D. addition of anti-mitotic cocktail

Cultures were incubated at 37 ℃, with 5% CO_2. 24 h later, 150 mL of NGF stock solution (40 mM of 5-fluorodeoxyuridine and uridine, 1 mM Arabinofur-anosyl Cytidine (Ara C, Sigma-Aldich, Saint Louis, MO) in NG medium) was added into each well to make the final concentration of 5-fluorodeoxyuridine/uridine 20 mM. Cultures were incubated at 37 ℃ in a 5% CO_2 incubator. After 72 hours, 2/3 of the NGF stock solution was changed to NG medium and the cultures were re-fed every 2 days with NG medium. After three medium changes, the neurons were ready for the Schwann cell addition [3].

E. addition of Schwann cells into DRG neuronal cell culture

Schwann cells were digested and washed once with DMEM (Invitrogen, Carlsbad, CA) containing 10% FBS, and then resuspended in C medium (MEM, 10% FBS, 2mM l-glutamine, 0.4% glucose, and 50 ng/mL 2.5 S NGF). 100 mL (approximately 200,000 cells) of Schwann cells was added to each of the DRG cultures in C media. After 3 days, the co-cultures were supplemented with 50 mg/mL of ascorbic acid to initiate basal lamina formation and myelination. Myelination was allowed to proceed for up to 21 days. The process of myelination was observed and recorded under phase contrast microscope.

F. Immunocytochemistry

To observed the formation of the myelin, the DRG neuron/SC co-cultures (14 days after initiation myelin formation) were then labeled with primary antibodies, which included monoclonal antibody (mAb) against MPZ. After three washes with PBS, the coverslips were further incubated with Alexa Fluor 488 mouse anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA) for 60 min at room temperature. After a final wash in PBS, the slides were mounted with mounting fluid (DAKO Ltd., Carpenteria, CA) and visualized under a fluorescence microscope (Olympus, Tokyo, Japan). The images were digitally recorded and processed with Image-Pro Plus (Media Cybernetics, Atlanta, GA).

G. Transmission Electron microscopy

Samples was fixed for 12hours in 2% Glutaraldehyde Paraformaldehyde in 0.1Mphosphate buffer(pH 7.4) and washing in 0.1M phosphate buffer. They were postfixed with 1% OsO4 dissolved in 0.1M PB for 2hr and dehydrated in ascending gradual series(50 ~ 100%) of ethanol and infiltrated with propylene oxide. Specimens were embedded by Poly/Bed 812 kit (Polysciences). After pure fresh resin embedding and polymerization at 65℃ electron microscope oven(TD-700, DOSAKA, Japan) for 24hr.

III. Results and conclusions

This procedure contains following four steps: first step of suspension of the embryonic dorsal root ganglion cells, secondstep of addition of anti-mitoticcocktail, third step of purification of dorsal root cells, and fourth step of addition of Schwann cells to dorsal root ganglion cells.

As a result of this study, for formation of myelination, Schwann cells and neuronal cells,

respectively, were prepared and cultured from DRG of rat embryos (E 16 day) (Figure 1).

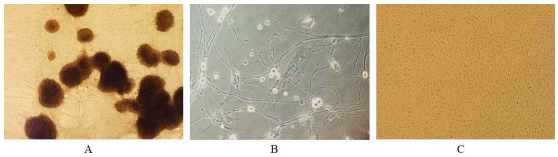

Figure 1. Populations of Schwann cells and neuronal cells, respectively, isolated and purified from DRG of rat embryo (E 16 day) (A: DRG; B: neuronal cells; C: Schwann cells).

To identify myelination and demyelination processing, population of cells were labeled with monoclonal antibody against MPZ and observed by fluorescent microscope. Population of myelinated cells represent fluorescent spots due to monoclonal antibody against MPZ which binds myelinated proteins. On the other hand, population of demyelinated cells did not because of absence of monoclonal antibody against MPZ (Figure 2).

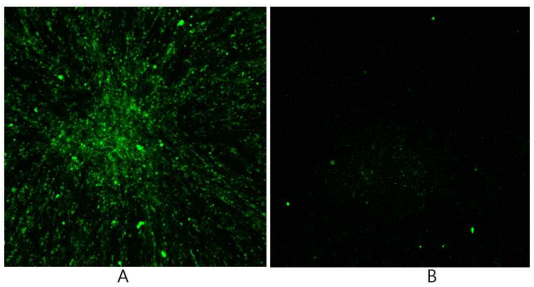

Figure 2. Identification of demyelination processing with polyclonal antibody against MPZ protein after infection of sindbis virus into DRG neuron and Schwann co-culture (A: population of myelinated cells by fluorescent microscope; B: population of demyelinated cells by fluorescent microscope).

On the other hand, we can identify myelination and demyelination processing after infection of sindbis virus into co-culture with DRG neuron and Schwann cells (Figure 3).

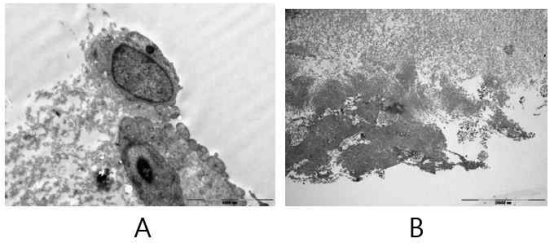

Figure 3. Identification by electron microscope on myelination and demyelination processing after infection of HSV-1 into DRG neuron and Schwann co-culture (A: population of myelinated cells; B: population of demyelinated cells).

The purpose of this study was to investigate the developmental process of myelination by neuron and Schwann cell cultures and the development of demyelination by herpes simplex virus-1 infection by electron microscopy and molecular biological analysis. The dorsal root ganglion (DRG) was isolated from the mouse embryo and Schwann cells and neuronal cells were cultured in vitro. Neuronal cells treated with mitotic inhibitors and purified Schwann cells were co-cultured together to induce myelination. The herpes simplex virus-1 was infected with the co-cultured cells, and the demyelination was induced. The myelin protein zero (MPZ) antibody, which means the presence of myelin formation, was used and electron microscopy was used to observe the development of myelin and dehydration.

References

[1] https://en.wikipedia.org/wiki/Demyelinating_disease

[2] M. Zandian, K. R. Mott, S. J. Allen, O. Dumitrascu, J. Z. Kuo and H. Ghiasi, “Use of cytokine immunotherapy to block CNS demyelination induced by a recombinant HSV-1 expressing IL-2,”*Gene Therapy* vol. 18, pp. 734–742, 2011.

[3] R. Liu, G. Lin, and H. Xu, “An efficient method for dorsal root ganglia neurons purification with a one-time anti-mitotic reagent treatment,” *PLoS one* vol. 8, pp. 1-7, 2013.

[4] J. Fukuda, “Nerve cells of adult and aged mice grown in a monolayer culture: age-associated changes in morphological and physiological properties of dorsal root ganglion

cells in vitro," *Develop. Neurosci.* vol. 7, pp. 374-394, 1985.

[5] J. Fukuda, and M. Kameyama, "Enhancement of Ca spikes in nerve cells of adult mammals during neurite growth in tissue culture," *Nature,* vol. 279, pp. 546-548, 1979.

[6] K. Unsicker, S. D. Skaper, G. E. Davis, M. Manthorpe, S. Varon, "Comparison of the effects of laminin and the polyornithine-binding neurite promoting factor from RN22 Schwannoma cells on neurite regeneration from cultured newborn and adult rat dorsal root ganglion neurons,"Developmental Brain Research vol. 7, pp. 304-308, 1985.

[7] H. A. Kim, and P. Maurel, "Protocols for Neural Cell Culture, Springer Protocols Handbooks"Humana Press, a part of Springer Science+Business Media, pp. 253−268, 2001.