Sindbis Virus에 의한 뉴런세포의 탈수초의 유도

사영희* · 김현주* · 권태동* · 김지영* · 이배환** · 홍성갑***

*,**연세대학교 의과대학교 마취통증학과, ***목원대학교 바이오건강학부

Induction of Demyelination of Neuronal cells by Sindbis Virus

Young-Hee Sa* · Hyun Joo Kim* · Tae Dong Kweon* · Ji-Young Kim* ·

Bae Hwan Lee** · Seong-Karp Hong***

*Department of Anesthesiology and Pain Medicine,

Yonsei University College of Medicine, Seoul, 03722, South Korea

**Department of Physiology and Brain Korea 21 PLUS Project for Medical Science,

Yonsei University College of Medicine, Seoul 03722, Korea

***Division of Bio and Health Sciences, Mokwon University, Daejeon, 35349, South Korea

E-mail : karp@mokwon.ac.kr

요 약

마우스 간염 바이러스, 코로나, 홍역 및 sindbis 바이러스와 같은 많은 바이러스가 쥐의 신경계에서 수초 형성의 파괴를 의미하는 탈수 초 유도의 원인 바이러스로 알려져 있다. 본 연구의 목적은 슈반 세포와 신경 세포의 공동 배양에 의한 수초화와 쥐의 sindbis 바이러스 감염에 의한 탈수초화에 의한 수초화 과정을 연구하는 데 있다. 쥐의 배아의 (Dorsal root ganglion, DRG)에서 슈반 (Schwann) 세포와 신경 세포 (neuronal cell)를 *in vitro*에서 배양 하였다. 유사 분열 억제인자로 처리한 신경세포와 정제 된 Schwann 세포를 갖는 공동 배양을 하였다. 그 후,이 수초화 된 공동 배양 시스템에 sindbis 바이러스 감염이 수행되었다. 수초 형성의 존재를 의미하는 peripheral myelin protein 22 (PMP 22) 항체를 사용하여 수초 형성 및 탈수초화 과정을 관찰 하였다. 우리는 수초화 된 뉴런의 존재를 의미하는 말초 myelin 단백질 22 (PMP 22)의 항체를 사용하여 수초화 및 탈수초 과정을 확인하였다. 이 연구는 과학 기술부, ICT 및 미래 계획 (NRF-2015R1C1A1A01053484 및 2017R1A2B3005753)이 자금을 지원하는 국립 연구 재단 (NRF)을 통한 기초 연구 프로그램의 지원을 받았다.

ABSTRACT

Many viruses including mouse hepatitis virus, corona, measles, and sidbis viruses are known as causative virus of inducing demyelination which means destruction of myelination in nervous system of mice. The purpose of this study is to investigate processing of myelination by co-culture of Schwann cells and neuronal cells and demyelination induced by infection of sindbis virusin rat. Schwann cells and neuronal cells from dorsal root ganglion (DRG) in embryos (E16) of rat were cultured in vitro respectively. The purified neuronal cells with anti-mitotic agents and purified Schwann cells were co-cultured. After that, infection of sindbis virus into this myelinated co-culture system was performed. Myelination and demyelination process were observed using antibody of myelin basic protein meaning presence of myelination.We identified myelination and demyelination processing using antibody of peripheral myelin protein 22 (PMP 22) meaning presence of myelinated neuron. This study was supported by the Basic Research Program through the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2015R1C1A1A01053484 and 2017R1A2B3005753).

키워드

수초, 탈수초, peripheral myelin protein 22 항체, sindbis virus

Ⅰ. INTRODUCTION

Sindbis virus is a member of the Togaviridae family, in the alphavirus subfamily. Sindbis virus causes sindbis fever in humans and the symptoms include arthralgia, rash and malaise. Sindbis virus

is an "arbovirus" (arthropod-borne) and is maintained in nature by transmission between vertebrate (bird) hosts and invertebrate (mosquito) vectors. Humans are infected with Sindbis virus when bitten by an infected mosquito. Sindbis virus has been linked to Pogosta disease in Finland, Ockelbo disease in Sweden and Karelian fever in Russia.

Researchers developed a DRG neuron and schuwann cells co-culture system for myelin research *in vitro* [1]. 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 [2, 3, 4].

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.

From this research, we constructed a population of myelinated cells with co-culture of neuronal cells and Schwann cells from DRG. After this myelinated cells were infected with sindbis forest virus and processing of demyelination was progressed. We could identify and distinguish myelination and demyelination processing using antibody of PMP22 which represented as myelinated cells.

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[5]. 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[2].

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 PMP22. 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).

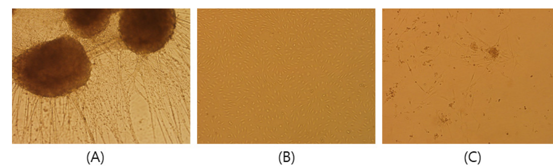

Figure 1. Purfication of populations of Schwann cells and neuronal cells, respectively, from DRG of rat embryo (E 16 day) (A: DRG; B: Schwann cells; C: neuronal cells).

To identify myelination and demyelination processing, population of cells were labeled with monoclonal antibody against PMP22 and observed by fluorescent microscope. Population of myelinated cells represent fluorescent spots due to monoclonal antibody against PMP22 which binds myelinated proteins. On the other hand, population of demyelinated cells did not because of absence of monoclonal antibody against PMP22 (Figure 2).

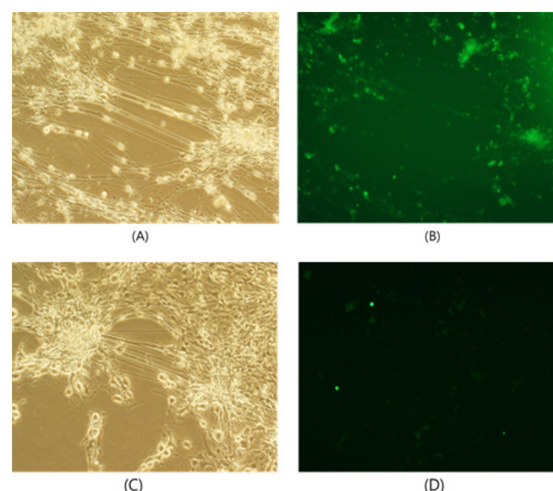

Figure 2. Identification and distinction of myelination and demyelination processing with monoclonal antibody against PMP22 (A: population of myelinated cells, B: anti-PMP22 in myelinated cells C: population of demyelinated cells, D: anti-PMP22 in demyelinated cells).

In the meanwhile, we can confirm population of myelinated cells by transmission electron microscopy (Figure 3).

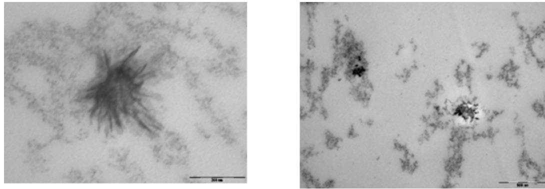

Figure 3. Photographs of Transmission Electron microscopy of population of myelinated cells

In this study, myelination had been constructed by co-culturing with pure populations of primary Schwann cells and primary neuronal cells, respectively. These co-culture populations of primary Schwann cells and primary neuronal cells, respectively, were driven from DRG of rat embryos. When sindbis virus infected co-culture populations and broke down myelination processing through acting to nerve cells. Presence of monoclonal antibody (labelled with fluorescent dye) against PMP22 indicates presence of myelinated cells. Moreover, we recognize that infection with sindbis virus induces demyelination processing.

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