Caspian Journal of Neurological Sciences
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Administration of Leukemia Inhibitory Factor Increases Opalin Expression in the Cerebral Cortex of Male Balb/C Mice; An *In Vivo* Study

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ARTICLE INFO

Article type:
Original Article

Article history:
Received: 19 April 2015
Accepted: 23 June 2015
Available online: 9 July 2015
CJNS 2015; 1 (2): 1-7

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ABSTRACT

Background: Leukemia inhibitory factor (LIF) is a neurortophic cytokine which plays an important role in the neural cell survival. Expression of LIF and its receptor, LIFR, in different brain regions has been demonstrated. Based on evidences LIF plays an important role in the modulation of neurogenesis and glial responses to injury. Up-regulation of LIF after central nervous system (CNS) damage is an endogenous response that limits injury by a direct protection of neurons and oligodendrocytes. More importantly LIF is an important cytokine that stimulates oligodendrocyte proliferation *in vitro*. Also Opalin is a unique molecular marker of mature oligodendrocyte. It is a transmembrane protein which is specifically expressed by myelinating oligodendrocytes.
Objectives: The aim of this study was to investigate the effects of LIF on Opalin expression in the Balb/c mouse cerebral cortex.
Materials and Methods: LIF was administered intraperitoneally (IP) to 6 to 8 weeks mice. The second group (SHAM) was injected IP by normal saline and third group was left without injection as the control group (n=9 for each group). One day after injection, the mice were killed by overdose of anaesthetic (sodium pentobarbitone) and brains were removed for further analysis.
Results: Using Western blotting we showed that administration of LIF increases Opalin expression in the cerebral cortex extracts.
Conclusion: The results from this study suggest that LIF increases Opalin expression in the cerebral cortex *in vivo*.

Keywords: Leukemia Inhibitory Factor; Opalin; Cerebral Cortex

➢ ***Please cite this paper as:***
Mashayekhi F, Salehi Z, Eslami M, Rajaei F. Administration of Leukemia Inhibitory Factor Increases Opalin Expression in the Cerebral Cortex of Male Balb/C Mice; An *In Vivo* Study. Caspian J Neurol Sci 2015; 1(2):1-7.

Introduction

The myelin sheath is a multilamellar membrane structure formed by myelin-forming glial cells, oligodendrocyte and schwann cells in the central nervous system (CNS) and peripheral nervous system (PNS), respectively (1). The biogenesis of the myelin membrane requires intricate machinery: the glial cells have to associate with the axons at the appropriate developmental time (2). Myelin basic protein

(MBP) and proteolipid protein (PLP) are two major protein constituents in CNS myelin (3). During development, oligodendrocyte progenitor cells (OPCs) differentiate into pre-oligodendrocytes and subsequently into immature and pro-myelinated oligodendrocytes, which then can myelinate the axons (4). Although the process of differentiation of OPCs has been investigated in terms of the spatially and temporally restricted expression of some myelin-constituting genes (5), the molecular mechanism of oligodendrocyte-directed gene expression is not fully understood.

It has been shown that one of the identified proteins, transmembrane protein 10 (Tmem10; also known as Opalin or oligodendrocyte paranodal loop protein), whose expression is specific to the brain, predominantly exists in many white matter regions and will be up-regulated during the postnatal developmental stages (6). It was shown that Opalin protein is specifically expressed by myelinating oligodendrocytes, suggesting that it plays a unique role in mammalian myelin. Opalin which has the approximate molecular mass of 38.5 kilo Dalton is specifically localized in CNS myelin (7).

Growth factor expression could participate in the repair process of the demyelinating disease by modulating the activity of microglia/macrophages in an autocrine fashion, by inducing the expression of other factors that can affect myelin regeneration or degeneration, and also by directly stimulating the localized proliferation and/or regeneration of oligodendrocytes within lesioned areas (8). Among growth factors, leukemia inhibitory factor (LIF) is a cytokine that has been demonstrated to play an important role in the neural cell survival including cholinergic neurons (9). It has been demonstrated that LIF limits autoimmune demyelination and oligodendrocyte loss in a murine model of multiple sclerosis (MS) (10). At later stages of development, LIF can act as a survival and proliferation factor for primordial germ cells (11). Cranial mesencephalic motor neurons increase choline acetyltransferase in response to LIF (12). It is suggested that LIF has both reparative and protective activities that make it a promising potential therapy for CNS demyelinating disorders and injuries (13). LIF was also shown to be a survival factor for sensory neuron (14). Spinal cord precursors synthesize LIF, and anti-LIFRβ antibodies completely blocked the generation of neurons. LIF increases the number of GFAP-positive cells *in vitro*. LIF promotes the differentiation of either neurons or astrocytes depending on the culture conditions (15). LIF binds to a heterodimeric membrane receptor complex consisting of LIF receptor (LIFR) and glycoprotein 130 (gp120) (16). This receptor is now termed LIFRβ and it binds to LIF with low affinity. LIF binds with high affinity to a LIFRβ/gp130 complex, which also results in signal transduction (17). Cooperative expression of LIF and LIFR in the brain of patients with neurodegenerative disease including Parkinson's disease (PD) and Alzheimer's disease (AD) may indicate the role of LIF in neuronal damage or repair in these diseases (18). It has been shown that LIF is a neurotrophic cytokine and plays an important role in myelin formation *in vitro* (19,10), which led us to examine the *in vivo* effects of this growth factor on Opalin expression in the mouse cerebral cortex.

Materials and Methods

Animals

Balb/c mice were maintained on 12-12 light: dark cycle beginning at 8.00 a.m. They were kept at a constant temperature in mice boxes with unrestricted access to laboratory food and water. The colony was maintained through random pair mating. All animal procedures were carried out in accordance with the Animals (Scientific Procedure) Act, 1986. All animal protocols used have been approved by the authors' institutional animal experimentation committee. The number of 27 male Balb/c mice with the postnatal age of 6 to 8 weeks were included in this study (n=9 for each group).

In vivo growth factor treatment

The first group of male Bulb/c mice were injected intraperitoneally (IP) with 100 ng mouse LIF (Chemicon, CA) in 0.1 ml sterile PBS. The second group (SHAM) was injected IP by normal saline in the amount of 0.1 ml and third group was left without injection as the control group. One day after injection, the pups were collected after euthanasia, by intraperitoneal injection of an overdose of anesthetic (Sodium Pentobarbitone) and the cerebral cortex were removed and processed as described below. In each of the experimental groups the number of animals investigated was n=9.

Cell extract

Frozen cerebral cortical tissue samples (12 mg each) were chopped into tiny pieces and suspended in 0.5 ml of protein lysis buffer [150 mM NaCl, 1.0% NP40, 20 mM Tris (pH 7.5), 5mM EDTA, and Complete Mini protease inhibitor cocktail (Roche Diagnostics Ltd., West Sussex, UK)] and then mechanically homogenized by sonication. After centrifugation, the protein extracts were collected and stored at -70°C until they were used.

Total protein concentration and western blotting

The total protein concentration in cerebral cortex extracts was determined by the Bio-Rad protein assay based on the Bradford dye procedure. For Western blot, protein extracts (50µg/lane) were separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Ltd. Hertfordshire, UK). The membranes were blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 5% dry milk and probed either with polyclonal anti- Opalin antibody (Sigma-Aldrich; HPA014372) (1:500 dilution) or a mouse monoclonal anti-β-tubulin antibody (Abcam plc, Cambridge, UK) (1:10,000 dilution) and then treated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive protein was visualized using the Enhanced Chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis was performed by scanning immunoblots and quantitating protein bands using an image analyzer.

Statistical analysis

All data presented are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the one-way ANOVA to test for differences among the groups, and only values with $p \leq 0.05$ were considered as significant.

Results

Total protein concentration

The total protein concentration in the cerebral cortex extracts from LIF injected and control animals were determined by the Bio-Rad protein assay based on the Bradford dye mixture. The total protein contents of controls, SHAM and LIF injected was 0.97±0.11, 0.98±0.14 and 0.99±0.16 (g/l), respectively. No significant increase in the total protein concentration was seen in the LIF-injected cerebral cortex samples compared with those from control and SHAM groups of the same age (p>0.05) (Diagram 1).

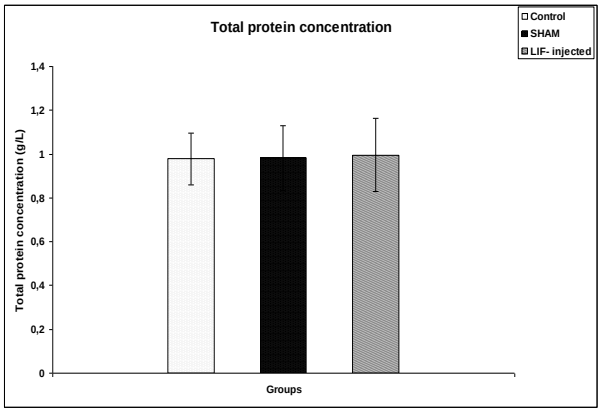

Diagram 1: Total protein concentration in the cerebral cortex extracts from control, SHAM and LIF-injected groups (g/L). No significant difference has been seen in total protein concentration between the groups (p > 0.05). In each of the experimental groups the number of animals investigated was n= 9.

Analysis of Opalin western blotting

Western blot analysis was performed to quantitatively evaluate Opalin expression in the cerebral cortex extracts. A Western blot analysis using anti-Opalin antibody as a probe confirmed the presence of Opalin (Figure 1).

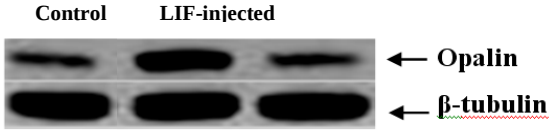

Figure 1: Expression of Opalin (molecular mass of 38.5 kDa) in the cerebral cortex extracts from control, SHAM and LIF-injected mice. β-tubulin (50-kDa) expression was determined as a protein loading control.

An image analyzer was used to determine the intensities of the band in the respective lanes. Quantification of the Western blot bands from repeated experiments (n=9) showed that the amount of Opalin was significantly increased in the LIF-injected cerebral cortex extracts when compared with control and SHAM groups (3.05 and 2.98 folds, respectively) (p < 0.0001) (Diagram 2). However, no significant change in the Opalin expression was seen between SHAM and control groups (p = 0.58).

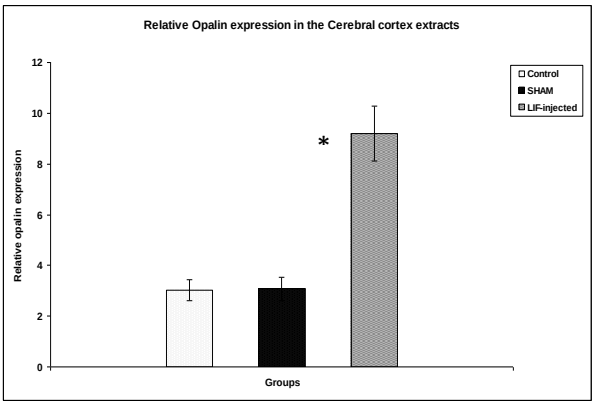

Diagram 2: Signal intensities from the control, SHAM and LIF-injected immunoblotting experiments were determined by densitometric analysis. Significant increase in the Opalin expression was seen between LIF-injected versus control and SHAM groups groups (3.05 and 2.98 fold, respectively) (p < 0.0001). However no significant difference was seen between SHAM and control group (p = 0.58). ***p < 0.001.

Discussion

The finding of this study showed that the administration of LIF increases Opalin expression in the cerebral cortex. LIF has been demonstrated to have beneficial effects on oligodendrocyte and exogenous LIF has therapeutic potential in limiting the consequences of oligodendrocyte damage (19). It has been suggested that LIF signaling cascades play significant roles in the differentiation of oligodendrocytes (20). It was shown that LIF has both reparative and protective activities that make it a promising

potential therapy for CNS demyelinating disorders and injuries (13).

Although the present study resulted that LIF increases Opalin expression in the cerebral cortex, however no significant change was seen in the total protein concentration in the cerebral cortex between the groups. Opalin is specifically expressed by myelinating oligodendrocytes and plays a key role in CNS myelination (7).

Acute demyelination of the CNS in multiple sclerosis (MS) is initially followed by a process of remyelination (21). This repair process is guaranteed by the extensive proliferation of oligodendrocyte progenitor cells (OPCs) in response to demyelination. It has been demonstrated that, growth factor expression could be important in the repair process of this demyelinating disease by modulating the activity of microglia /macrophages by inducing the expression of other factors that can affect myelin regeneration and also by directly stimulating the localized proliferation and/or regeneration of oligodendrocytes within lesioned areas (8).

Among growth factors, LIF has been shown to play an important role in the neural cell survival (22). LIF plays an important role in generation, maturation and survival of oligodendrocyte (23). Data supporting a neuroprotective role of LIF has been obtained in MS, where LIF has been demonstrated to be produced by myelin-reactive T cells isolated from MS patients and protect against tumor necrosis factor-α-induced oligodendrocyte apoptosis (24).

We investigated LIF as it is one of the most important growth factor involved in the survival of oligodendrocytes (25). LIF contributes in the differentiation of glial cell lineage (26). LIFR has been shown to be expressed in the dorsal ventricular zone and subventricular zone of the cerebral cortex (27). Our findings showed that the level of Opalin increases in response to LIF injection compared to controls. We have also shown that there is no significant change in the total protein concentration in the cerebral cortex between the groups. Many proteins are present in the mouse cerebral cortex extract and a change in one protein does not basically change the overall protein concentration.

Conclusion

It is concluded that LIF increases Opalin expression in the mouse cerebral cortex *in vivo*. It additionally re-emphasizes the importance of further investigation into the potential roles of LIF on gene expression in the cerebral cortex.

Acknowledgements

This study was supported partly by the Guilan University. The authors thank alla in the Genetics and Developmental Biology laboratories, Department of Biology, Faculty of Sciences, Guilan University, for their technical assistance.

Conflict of Interest

No Conflict of Interest.

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