Neuroscience 269 (2014) 1–10

BLOCKADE OF IL-6 SIGNALING BY MR16-1 INHIBITS REDUCTION OF DOCOSAHEXAENOIC ACID-CONTAINING PHOSPHATIDYLCHOLINE LEVELS IN A MOUSE MODEL OF SPINAL CORD INJURY

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Abstract—The interleukin (IL)-6 pathway plays an important role in recovery after spinal cord injury (SCI). The anti-IL-6 receptor antibody MR16-1 has been shown to suppress inflammation after SCI and promote recovery of motor function. The purpose of this study was to analyze the effects of MR16-1 on the expression patterns of phospholipids in the spinal cord in a mouse model of SCI. Eight-week-old C57BL/6JJmsSlc mice were used in this study. Laminectomy was performed at the ninth and tenth thoracic levels (T9–T10), and contusion injury of the spinal cord was induced at level T10. Immediately after SCI, mice were intraperitoneally injected with a single dose of MR16-1 (MR16-1 group) or a single dose of phosphate-buffered saline of the same volume (control group). Imaging mass spectrometry was performed to visualize phosphatidylcholine (PC) expression in the spinal cord 7 days after SCI. We found that MR16-1 treatment suppressed the infiltration of immune cells after SCI, and was able to increase the locomotor function post-injury. Phospholipid imaging revealed that the MR16-1 was able to prevent the reduction of docosahexaenoic acid (DHA)-containing PC in comparison with the control group. We also observed high levels of glial fibrillary acidic protein (GFAP) at the site of DHA-containing PC expression in the MR16-1 group. These results suggest that MR16-1 treatment influences the DHA-containing PC composition of GFAP-positive cells at the injury site as early as 7 days post-SCI.

Key words: spinal cord injury, IL-6, imaging mass spectrometry, phospholipids, docosahexaenoic acid, phosphatidylcholine.

INTRODUCTION

In recent years, despite the progresses in medical research, effective therapeutic approaches have not yet been established for treating injuries to the central nervous system (CNS). After CNS injury, axons are unable to regenerate beyond the injury site in mammals (Fawcett and Asher, 1999; Raineteau and Schwab, 2001; Fehlings, 2008; Fitch and Silver, 2008). After the initial injury that consists of neuronal and glial cell death, secondary pathology induced by inflammation occurs at the injury site (Hausmann, 2003; Donnelly and Popovich, 2008). Numerous studies have focused on neurotrophic factors to prevent secondary degeneration, with the aim of reducing the area of damage and promoting axonal regeneration through the lesion epicenter (Nesic et al., 2001; Sharma et al., 2003). Several recent reports suggest that inflammation can be beneficial or even essential for spinal cord repair, because it clears tissue debris and promotes the secretion of various neurotrophic factors (Hashimoto et al., 2005; Donnelly and Popovich, 2008). Accordingly, pro-inflammatory cytokines have been identified as promising targets for developing spinal cord injury (SCI) treatments (Nesic et al., 2001). Among these cytokines, interleukin (IL)-6 is known to markedly promote the activation and infiltration of macrophages/microglia (Hurst et al., 2001; Sharma et al., 2003). A humanized antibody for the human IL-6 receptor (MRA; tocilizumab) is currently in clinical use for the treatment of Castleman's disease and rheumatoid arthritis (Sato et al., 1993; Nishimoto et al., 2000; Choy et al., 2002). Another IL-6 receptor antibody, MR16-1 (Tamura et al., 1993), has proven to be effective in treating mouse models of SCI by improving motor function through its ability to reduce inflammation, decrease glial scar formation, and increase tissue sparing (Okada et al., 2004; Mukaino et al., 2010). However, recent studies using gene-knockout animals have revealed that the continuous inhibition of IL-6 hinders functional recovery, perhaps due to the inhibition of axonal regeneration or the absence of normal gliosis. Thus, studies performed in knockout animals suggest that IL-6 itself has a beneficial function in spinal cord repair (Cafferty et al., 2004; Okada et al., 2006). On the

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Abbreviations: AA, arachidonic acid; BMS, Basso Mouse Scale; DHA, docosahexaenoic acid; DHB, dihydroxybenzoic acid; FA, fatty acid; GFAP, glial fibrillary acidic protein; IL, interleukin; IMS, imaging mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SCI, spinal cord injury; SPSS, Statistical Package for the Social Sciences; TOF, time-of-flight.

http://dx.doi.org/10.1016/j.neuroscience.2014.03.012
0306-4522/

other hand, IL-6 cytokine family overexpression during the acute phase of SCI can significantly increase inflammatory cell accumulation, resulting in greater damage (Lacroix et al., 2002; Kerr and Patterson, 2004). Considering these contrasting findings, an improved understanding of alterations in the spinal cord after MR16-1 treatment could shed some light on the underlying role of IL-6 in recovery after SCI.

The most common biomolecules in the CNS are lipids, which are involved in diverse cellular functions. In particular, lipids are important for regulating the physical properties of cellular membranes (Rohrbough and Broadie, 2005; Takamori et al., 2006; Piomelli et al., 2007; Jacobson et al., 2007) and for signal transmission mediated by bioactive lipids such as prostaglandins (PGs; Williams et al., 1989; Dinh et al., 2002; Piomelli et al., 2007). Moreover, alterations in lipid metabolism may contribute to physiological responses observed following CNS injury. For example, lipids such as PGs are produced by invasive immune cells and have been linked to severe inflammation in the injured spinal cord (Hermann et al., 2001; Buczynski et al., 2010; Hanada et al., 2012). Similarly, elevated levels of the lysophospholipid group have been observed post-SCI (Ma et al., 2010). These results emphasize the important contributions of lipid dynamics to processes involved in tissue inflammation (Ma et al., 2010; Buczynski et al., 2010; Girod et al., 2011). Biochemical quantification of lipids in fluid samples of cellular or tissue extracts has traditionally been the mainstay of lipid research. Recently, imaging mass spectrometry (IMS) has emerged as a promising new technology that allows spatially resolved imaging of lipid distribution *in situ* (Hayasaka et al., 2008; Sugiura and Setou, 2010).

Lipid research, in particular, has greatly benefited from IMS by incorporating the technology with matrix-assisted laser desorption/ionization (MALDI) (Garrett and Yost, 2006; Cornett et al., 2007; Jackson and Woods, 2009; Ma et al., 2010; Sugiura and Setou, 2010). One major advantage of IMS is that it allows simultaneous detection of multiple lipids in the same tissue sample; therefore, lipid molecules with slight structural differences can be distinguished and heterogeneous distribution in biological tissues can be visualized (Touboul et al., 2005; Murphy et al., 2009; Sugiura et al., 2009; Sugiura and Setou, 2010; Girod et al., 2011). Additional advantages of IMS are that the protocol is simple, quick, and can be used on many different lipid classes (Cornett et al., 2007; Sugiura et al., 2008, 2011; Delvolve et al., 2011). In fact, using rat models of SCI, recent IMS studies have identified specific lipid species that accumulate in the approximate lesion area post-injury (Girod et al., 2011; Hanada et al., 2012).

The purpose of this study was to analyze alterations in lipids in the injured spinal cords of mice treated with MR16-1 to reveal the lipid pathophysiology involved in SCI.

EXPERIMENTAL PROCEDURES

Chemicals

Methanol, potassium acetate, and ultrapure water were purchased from Wako Chemicals (Osaka, Japan). The antibiotic Bactramin was purchased from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Calibration-standard peptides and 2,5-dihydroxybenzoic acid (DHB), a MALDI matrix, were purchased from Bruker Daltonics (Leipzig, Germany). All chemicals used in this study were of the highest purity available.

Abbreviations for glycerophospholipids

The structures of the side chains of glycerophospholipid species with one or two radial side chains are indicated within parentheses in the following format: head group (coupling scheme-sn1/sn2); e.g., phosphatidylcholine (PC) (diacyl-16:0/18:1).

Animals

We used 40 adult female C57BL/6JJmsSlc mice (8 weeks old) in this study. The procedures followed for the care and use of the laboratory animals were approved by the Ethics Committee of the Hamamatsu University School of Medicine. (Shizuoka, Japan).

Induction of SCI

Mice were deeply anesthetized with 25 mg/kg pentobarbital sodium, and laminectomy was performed at the ninth and tenth thoracic (T9–T10) levels. Briefly, the dorsal surface of the dura mater was exposed (taking care to avoid any dural tears), and the vertebral column was stabilized with fine forceps and clamps at T8 and T11. Subsequently, a moderate contusion injury of the spinal cord was induced at T10 using the commercially available Infinite Horizons impactor (IH impactor; Muromachi, Tokyo, Japan) with an impact force of 60 kdyn, as described previously (Okada et al., 2006; Mukaino et al., 2010; Guerrero et al., 2012). After contusion injury, the wound was closed by suturing the muscle layer using nylon sutures, and the skin was closed with wound clips. Mice that had undergone the SCI operation were kept in a temperature-controlled chamber until normal thermoregulation was reestablished. Antibiotics were given to animals from their drinking water (0.5 ml of Bactramin [Chugai] in 200 ml of water) for seven days post-SCI to prevent urinary tract infection. Manual bladder expression was performed every day until the reflex bladder emptying recovered. Additionally, residual urine was collected and the amount was recorded for 21 days post-injury. Food and water were provided *ad libitum*, and animals were housed three/cage in a room with a 12-h light/dark cycle.

MR16-1 treatment

Immediately after SCI, mice were intraperitoneally injected with a single dose of MR16-1 (Chugai; 100 μg/g body weight, MR16-1 group; $n = 19$) or a single dose of phosphate-buffered saline (PBS) of the same volume (control group; $n = 21$). Experimenters were blinded to the treatment groups throughout behavioral testing and histological analysis. To ensure unbiased evaluation, one surgeon performed all surgeries, while a surgical assistant randomly assigned the mice their ID numbers and treatment group designations.