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DIHYDROPYRIMIDINASE-LIKE 3 REGULATES THE INFLAMMATORY RESPONSE OF ACTIVATED MICROGLIA

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Abstract—Microglia, the resident immune cells of the CNS, are known to respond to injuries, infection and inflammation in the CNS by producing proinflammatory cytokines and phagocytosing cell debris and pathogens. In this study, we investigated the expression pattern and role of dihydropyrimidinase-like 3 (Dpysl3), a member of collapsin response mediator protein family, on the inflammatory reaction of microglia. Microarray analysis comparing the global gene expression profile of ameboid and ramified microglia has shown that Dpysl3 is mainly expressed in ameboid microglia in the 5-day postnatal rat brain. Immunohistochemical analysis revealed that Dpysl3 was intensely expressed in ameboid microglial cells in the rat brain till postnatal 7th day and then gradually diminished in ramified microglia of 2 weeks postnatal rat brain. Further, *in vitro* analysis confirmed that Dpysl3 expression was induced in activated BV-2 microglia treated with lipopolysaccharide (LPS). It is well documented that microglial activation by LPS increased the expression of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity in BV-2 microglia. However, siRNA-mediated knockdown of Dpysl3 prevented the LPS-induced expression of iNOS and cytokines including interleukin-1 beta, and tumor necrosis factor-alpha as well as nuclear translocation of NF-κB in microglia. Remarkably, knockdown of Dpysl3 inhibited the migration of activated microglia coupled with deranged actin filament configuration (as revealed by F-actin cytoskeleton expression) in lamellipodia projecting from the cells. Knockdown of Dpysl3 also inhibited the phagocytic ability of activated microglia. These findings suggest that knockdown of Dpysl3 can inhibit activation, migration and phagocytic capability of microglia and consequently reduce neuroinflammation.

INTRODUCTION

Microglia are the resident immune cells that mediate neuroinflammation in the central nervous system (CNS) (Ling et al., 2001). Morphologically, microglial cells in the CNS consist of two distinct phenotypes: the ameboid microglial cells (AMC) and ramified microglial cells (RMC). The AMC, which are abundant in the corpus callosum of the developing brain of neonatal rat pups, gradually transform into RMC with advancing age (Ling and Wong, 1993; Parakalan et al., 2012). Both AMC and RMC are activated by infection, trauma or any other pathological insult and the activated microglia perform diverse functions like clearing the toxic cellular debris via phagocytosis, production of proinflammatory cytokines and enhancing neuronal survival by the release of trophic factors (Dheen et al., 2007; Graeber and Streit, 2010). Chronic activation of microglia results in excessive release of proinflammatory cytokines and nitric oxide (NO) causing neurotoxicity (Block et al., 2007). The activated microglia undergo morphological transformation which enables them to be highly motile and phagocytic (Abdelbasset and Fedoroff, 1995). Further, it has been widely shown that the activated microglia exhibit two phenotypes: M1, which mediates inflammatory response and M2, which shows anti-inflammatory properties including scavenging of debris, tissue remodeling and repair (Gaikwad and Heneka, 2013; Lively and Schlichter, 2013).

Collapsin response mediator proteins (CRMPs) consist of five cytoplasmic phosphoproteins (CRMPs1–5) (Quinn et al., 1999, 2003) and each member of the CRMPs has been shown to be differentially expressed in the rat brain. The CRMPs play important roles in neuronal migration, synapse formation, synaptic plasticity, neuronal development and disease (Charrier et al., 2006; Niisato et al., 2012; Yamashita and Goshima, 2012). Further, the expression of CRMPs is dysregulated in neurodegenerative diseases such as Huntington's disease and Alzheimer's disease, indicating their roles in neuropathology (Jin et al., 2004; Cole et al., 2007). CRMP4, also known as dihydropyrimidinase-like protein 3 (Dpysl3), has been shown to be involved in neurogenesis during

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Abbreviations: AMC, ameboid microglial cells; CNS, central nervous system; CRMPs, collapsin response mediator proteins; DAPI, 4′,6-diamidino-2-phenylindol; Dpysl3, dihydropyrimidinase like 3; F-actin, filamentous actin; IL-1β, interleukin-1 beta; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; PBS, Phosphate-buffered saline; RMC, ramified microglial cells; RT-PCR, Reverse Transcription–Polymerase Chain Reaction; TNF-α, tumor necrosis factor-alpha; siRNA, small interfering RNA.

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development, neuroregeneration after nerve lesion and growth cone collapse during neuronal cell injury by organizing filamentous actin (F-actin) into tight bundles (Goshima et al., 1995; Minturn et al., 1995; Yuasa-Kawada et al., 2003). Studies show that Dpysl3 interacts with cytoskeletal proteins, tubulin and actin, suggesting its role in cell assembly and migration (Franken et al., 2003; Rosslenbroich et al., 2005). Recently, microarray analysis comparing the global gene expression profile of ameboid and ramified microglia showed that Dpysl3 is highly expressed in ameboid microglia of 5-day postnatal rat brain (Parakalan et al., 2012). However, the temporal expression pattern and roles of Dpysl3 in activated microglial cells have not been demonstrated previously.

In this study, we have further investigated the expression pattern of Dpysl3 and its role on microglial activation and neuroinflammation as this protein appears to have a role on cell assembly and migration. Immunofluorescence studies revealed its spatial and temporal expression pattern in microglia and small interfering RNA (siRNA)-mediated knockdown unraveled its role on microglia activation and migration. Overall, this study demonstrated that Dpysl3 is involved in activation, migration and phagocytic ability of microglia.

EXPERIMENTAL PROCEDURES

Animals

Wistar rats aged 5, 7, 14, 21 and 28 days were used in the study. Animal handling was in accordance to Institutional Animal Care and Use Committee (IACUC), National University of Singapore (NUS). Rats of 5 and 28 days of age were given an intraperitoneal (i.p.) injection of either 100 μl lipopolysaccharide (LPS) (1 mg/kg, Cat. L2654, Sigma–Aldrich, MO, USA) or equal volume of saline (control rats). The rats were sacrificed 6 h post injection and used for various experiments. For immunohistochemical analysis, rats were anesthetized with sodium pentobarbital solution (60 mg/kg), and then perfused transcardially with 2% paraformaldehyde in 0.1 M phosphate buffer. The brains were dissected out and post-fixed for 3 h in the same fixative, and then cryoprotected in 15% sucrose in 0.1 M phosphate buffer overnight. Frozen sections (20 μm in thickness) were cut coronally through the forebrain using a cryostat (Leica model 3050, Leica Instruments GmbH, NUBLOCH, Germany) and mounted onto gelatin-coated slides.

Perfusion of rats

Brain tissues used for immunofluorescence labeling of Dpysl3 were isolated from Wistar rats aged 3, 5, 7 and 14 days. The rats were anesthetized with sodium pentobarbital solution (60 mg/kg) and perfused with Ringer's solution and freshly prepared 2% paraformaldehyde. Brains were gently removed, kept in the fixative for 4 h and subsequently in 15% sucrose overnight at 4 °C. The brains were then rapidly frozen in liquid nitrogen and sectioned at 30-μm thickness using a cryostat.

BV-2 microglial cell culture

BV-2 cells, an immortalized murine microglial cell line, were used for *in vitro* study. The cells were grown in a 75 cm^2 cell culture flask at 37 °C in a humidified chamber filled with 5% CO_2 and 95% air for 3–4 days. Once reaching confluency, the cells were washed with phosphate-buffered saline (PBS) twice and detached by the addition of 5 ml of 1× trypsin–ethylenediaminetetraaceticacid for 5 min at 37 °C. The cells were transferred to Dulbecco's modified eagle medium (DMEM, Cat. D1152, Sigma, USA), supplemented with 10% fetal bovine serum (FBS, Cat. SV30160.03, HyClone, USA) and 1% antibiotic:antimycotic cocktail (complete medium). The complete medium containing BV-2 cells (20 ml) was centrifuged at 1000 rpm at 4 °C for 5 min and the pellet was resuspended in 10 ml of complete medium. The cells were then plated accordingly in 24-well cell plate for immunocytochemistry or plated in a 6-well plate for RNA isolation.

RNA isolation

Extraction of total RNA was carried out based on the instructions provided in the Qiagen RNeasy mini kit. Briefly, cells were lyzed in 650 μl of lysis buffer for 10–15 min on ice. The cell lysate was homogenized, before it was centrifuged for 30 s at 14000*g* in a microfuge. The subsequent steps were performed following the instructions of the Qiagen RNeasy mini kit. High-quality RNA obtained was then eluted in 20 μl of RNase free water. The concentration and purity of the extracted RNA was evaluated at 260 and 280 nm using a spectrophotometer.

Reverse Transcription–Polymerase Chain Reaction (RT–PCR)

cDNA was synthesized from the total RNA using the cDNA synthesis Kit (Promega, USA)and stored at −20 °C. Quantitative real time RT–PCR was performed in a thermocycler (ABI 7900HT, Applied Biosystems, Foster City, CA) using SYBR green I (dsDNA – specific binding dye for continuous fluorescent monitoring amplification). Each reaction mix contained 5 mM of each gene specific primer (1. Dpysl3 – Forward primer 5′-ATCAAGGGAGGGAGAATCGT-3′, Reverse primer 5′-TTGTCCCTTGGAAGAAATCG-3′; 2. TNF-α – Forward primer, 5′-CGTCAGCCGATTTGCTATCT-3′, Reverse primer 5′-CGGACTCCGCAAAGTCTAAG-3′; 3. IL-1β – Forward primer 5′-GCCCATCCTCTGTG ACTCAT-3′, Reverse primer 5′-AGGCCACAGGTATT TTGTCG-3′; 4. Inducible nitric oxide synthase (iNOS) – Forward primer 5′-GCTTGTCTCTGGGTCCTCTG-3′, Reverse primer 5′-CTCACTGGGACAGCACAGAA-3′; 5. β-Actin – Forward primer 5′-GGATTCCATACCCA AGAAGGA-3′; Reverse primer 5′-GAAGAGCTATGA GCTGCCTGA-3′), 2× PCR mastermix, and 1 μl of