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Caveolin isoform switching as a molecular, structural, and metabolic regulator of microglia ☆

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ABSTRACT

Microglia are ramified cells that serve as central nervous system (CNS) guardians, capable of proliferation, migration, and generation of inflammatory cytokines. In non-pathological states, these cells exhibit ramified morphology with processes intermingling with neurons and astrocytes. Under pathological conditions, they acquire a rounded amoeboid morphology and proliferative and migratory capabilities. Such morphological changes require cytoskeleton rearrangements. The molecular control points for polymerization states of microtubules and actin are still under investigation. Caveolins (Cavs), membrane/lipid raft proteins, are expressed in inflammatory cells, yet the role of caveolin isoforms in microglia physiology is debatable. We propose that caveolins provide a necessary control point in the regulation of cytoskeletal dynamics, and thus investigated a role for caveolins in microglia biology. We detected mRNA and protein for both Cav-1 and Cav-3. Cav-1 protein was significantly less and localized to plasmalemma (PM) and cytoplasmic vesicles (CVs) in the microglial inactive state, while the active (amoeboid-shaped) microglia exhibited increased Cav-1 expression. In contrast, Cav-3 was highly expressed in the inactive state and localized with cellular processes and perinuclear regions and was detected in active amoeboid microglia. Pharmacological manipulation of the cytoskeleton in the active or non-active state altered caveolin expression. Additionally, increased Cav-1 expression also increased mitochondrial respiration, suggesting possible regulatory roles in cell metabolism necessary to facilitate the morphological changes. The present findings strongly suggest that regulation of microglial morphology and activity are in part due to caveolin isoforms, providing promising novel therapeutic targets in CNS injury or disease.

Introduction

Microglia, resident central nervous system (CNS) macrophages, play an important role in the response to injury, infection and aging. Once considered to be "resting" or inactive cells, their ability to migrate and sense markers of injury and stress in the extracellular environment has led to a new understanding and appreciation of glia in response to CNS injury (Nimmerjahn et al., 2005). The central manifestation of the microglial response is to alter their morphology and migration to the site of injury, proliferate, and secrete cytotoxic or trophic molecules (i.e., the classical M1 macrophage type response). Chronically "activated" microglia result in neuroinflammation, typically exhibited in disease states such as Alzheimer's disease (AD), Parkinson's disease (PD) or multiple sclerosis (MS) (Glass et al., 2010). Though the M1 type activation of microglia has been extensively studied (Monif et al., 2010), reversal back to an inactive state has only recently been investigated (Larson et al., 2010; Lee et al., 2008; Watson et al., 2010).

Caveolae, a subset of membrane/lipid rafts (MLRs), are 50–70 nm plasmalemmal invaginations, highly enriched in sphingolipids and cholesterol, and the cholesterol binding protein caveolin; the latter of which serves to scaffold and dock many signaling proteins (Head and Insel, 2007; Patel et al., 2008). Three isoforms of caveolin (Cav-1, Cav-2 and Cav-3) are expressed in a variety of cells, with Cav-1 and Cav-2 predominantly expressed as heterodimers in vascular tissue, adipocytes and fibroblasts, while Cav-3 is highly expressed as a homodimer in muscle tissues (Chidlow and Sessa, 2010).

In regard to the CNS, all three isoforms have been described (Shin et al., 2005; Silva et al., 2007). Neurons, which are devoid of morphological caveolae, do express all three isoforms (Stern and Mermelstein, 2010). Astrocytes, neuronal supporting glia, show morphological caveolae and express all three isoforms (Ikezu et al., 1998). Peripheral macrophages

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have been found to express Cav-1 and Cav-2 (Kiss et al., 2002), yet microglial expression of caveolin has not been thoroughly investigated. Although Marella and colleagues (Marella et al., 2002) detected Cav-1 in a human microglia cell line, other reports have shown no Cav-1 expression in BV2 cells, a well-documented mouse microglia cell line (Park et al., 2009). Thus, the functional role of caveolin in microglia is still unknown.

Caveolins serve structural and metabolic roles in cells (Fridolfsson et al., 2012); two processes that may be key to microglial activation and inactivation. Polymerization and depolymerization of actin and tubulin are key regulators of cell morphology, organelle and vesicular transport, and cell migration. Cav-1 localization is dynamically regulated by both actin and tubulin in CHO cells (Mundy et al., 2002). An ultrastructural localization study of Cav-3 in splenic endothelial cells demonstrated an association with stress fibers determined to be mainly composed of actin (Uehara and Miyoshi, 2002). Regulation of the cell cytoskeleton directly by caveolins has been studied in other cell types for both Cav-1 (Shajahan et al., 2007) and Cav-3 (Head et al., 2006). With increasing awareness of the role of chronic inflammation after CNS injury or in neurodegenerative states (Cunningham, 2013; Nolan et al., 2013; Shultz et al., 2013), we investigated potential functional roles for Cav-1 and Cav-3 in regulation of microglial cell structure and metabolism. In this report, we define the expression of Cav-1 and Cav-3 in BV2 cells and in primary mouse derived microglia (PMG). Caveolins, which provide scaffolds for receptor signaling cascades as well as cytoskeletal tethering points at the PM, may serve an essential role for regulating early changes, both morphological and biochemical, in transitioning cell states between activation and inactivation of microglia. We have further defined potential key roles for caveolin in the regulation of mitochondrial function and cytoskeletal polymerization. Therefore, caveolin isoforms may represent potential regulators of microglial function and may also serve as a target for genetic or pharmacological manipulation in a variety of disease settings.

Results

In vitro model of microglial activation and inactivation

Microglia activation has been defined by proliferative potential, morphologic criteria, metabolic states, differential transcriptional regulation, and inflammatory markers (van Rossum and Hanisch, 2004). Cells were grown in either serum-free (HyC, homeostasis) or high serum conditions (D10%, constitutively active) prior to analysis of cellular morphology. GSA (*Griffonia simplicifolia*) lectin (red) and phalloidin (PHL — green) immunoflourescence (IF) labeling confirmed both the proliferation and morphological changes associated with culturing in the serum-free condition (HyC) or in 10% FBS (D10%) (Fig. 1A). HyC cells are highly processed and less numerous than D10% cells indicative of microglial inactivation and activation respectively. To quantify the morphological difference, cell fields (10 per group), were counted for processed cells from the GSA-PHL coverslips. HyC cells averaged 65–70% processes, while less than 10% of D10% cells exhibited extended processes (Fig. 1B). MTT assays assessed the proliferation of cells grown with high serum (D10%) or serum-free (HyC) media (Fig. 1C). Statistically significant growth differences were seen between HyC and D10% cells, with HyC grown cells numbering 40% less than D10% (**$p = 0.007$). Using cholera toxin (Ctx — green) as a marker for membrane/lipid rafts (MLR), BV2 cells were assessed for presence of MLR by IF microscopy (Fig. 1D). Cells grown in either media display Ctx labeled MLR. The cytoplasmic protein and microglial marker Iba1 (red) was used as a control for PM localization (Fig. 1E). By switching BV2 cells from an activation state/growth promoting media (D10%) to an inactivated and proliferation suppressing media (HyC), a model system has been established to study the transition from an inactive-homeostatic state to a constitutively active and highly proliferative cell phenotype.

Activation/inactivation of microglia results in distinct ultrastructural and metabolic profiles

Further morphological analysis was performed using routine electron microscopy to determine presence of morphological caveolae and to assess any ultrastructural difference between the two conditions (Fig. 1F: *small A–D*). Caveolae and clathrin coated pits (CCPs) were localized to the PM in both conditions with similar distribution and localization. D10% grown cells had a very dense endoplasmic reticulum and more abundant mitochondria (M) (Fig. 1F, *A*), suggestive of highly metabolic cells. HyC grown cells (Fig. 1F, *B*) contained a greater percentage of empty vacuoles and vacuoles with fusing vesicles and less numerous, smaller mitochondria, indicating a less active metabolic state. *C* and *D* are higher resolution images of PM areas focused on both CCP and caveolae in either condition. Metabolic activity was assessed with the Seahorse Bioscience metabolic flux analyzer. Both oxygen consumption rate (OCR, Fig. 1G) and extracellular acidification rate (ECAR, Fig. 1G), measures of mitochondrial respiration and glycolysis, respectively, were enhanced in D10% versus HyC grown cells. Such data suggest that the morphologic features observed in cells under various growth conditions impact metabolic function.

Cav-1 and -3 are differentially expressed with culture conditions and localize to discrete intracellular domains

IF microscopy of the BV2 cells grown in the different media exhibit different morphologies, and exhibit different expression levels and localization of Cav-1 and Cav-3 (Fig. 2A). HyC grown BV2 have low levels of Cav-1 (top left), while serum activated cells (D10%) have higher levels and show distinct punctate staining along the PM (left panels Fig. 2A). The middle columns of Fig. 2A provide the first evidence for the expression of Cav-3, normally associated with skeletal and cardiac muscle, in myeloid derived macrophage-like cells. In the highly processed cells (HyC), Cav-3 staining was associated with the cytosol, in perinuclear regions, as well as along cellular processes, the latter suggestive of a cytoskeletal distribution. Furthermore, both the perinuclear and total expression of Cav-3 were decreased as the cells transitioned from an inactive to an active state. We further assessed Cav isoforms in primary microglia (PMG) from neonatal mice (Fig. 2B). IF analysis of isolated PMG grown with either HyC or D10% and stained for Cav-1 and Cav-3 revealed similar findings seen in BV2 cells: in HyC media, PMG display extended processes with enhanced Cav-3 expression (red); D10% cells were more rounded or flattened with Cav-1 expression predominantly (green) at the PM and in the cytosol. However, in contrast to the highly compartmented expression in BV2 cells, some co-localization was detected in perinuclear regions in both media. To confirm these results, we used PCR in conjunction with Western blot (WB) assays to measure changes in caveolin isoform expression. HyC cells have 1.5 fold less Cav-1 mRNA ($p < 0.05$, $n = 3$ experiments) than D10% cells, but a 1.1 fold increase in Cav-3 mRNA ($p < 0.02$, $n = 3$ experiments) (Fig. 2C) in BV2 cells. Fig. 2C (right) shows qPCR products from wild type (WT) PMG grown in D10% for Cav-1 and Cav-3. The efficiency of Cav-1 and Cav-3 primers used for BV2 qPCR was not sufficient when used for PMG to acquire statistically significant quantitations but a reduction in Cav-1 mRNA was found for HyC to D10% (-1 ± 0.16 S.E.M. $\Delta\Delta CT$ $n = 4$) and an increase in mRNA for Cav-3 was found for HyC to D10% ($+1.74 \pm 0.31$ S.E.M. $\Delta\Delta CT$ $n = 4$). Protein expression and antibody specificity was determined by WB for WT, Cav-1 and Cav-3 KO PMG lysates, all grown in D10% to demonstrate antibody specificity (Fig. 2D). In BV2 cell lysates, Cav-1 showed the lowest expression in the inactive state (i.e., HyC); in contrast Cav-3 expression remained elevated (Fig. 2E). When PMG are cultured similarly to BV2 cells, HyC conditions also resulted in decreased Cav-1 and increased Cav-3 expression (Fig. 2F).

Microglia *in vivo* must be able to both activate and inactivate in response to a changing environment. To determine if the results of cells