Original Paper

Induction of Microglial Activation by Mediators Released from Mast Cells

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Key Words

HMC-1 • Microglial activation • CRH • Pro-inflammatory factors • MAPK

Abstract

Background/Aims: Microglia are the resident immune cells in the brain and play a pivotal role in immune surveillance in the central nervous system (CNS). Brain mast cells are activated in CNS disorders and induce the release of several mediators. Thus, brain mast cells, rather than microglia, are the “first responders” due to injury. However, the functional aspects of mast cell-microglia interactions remain uninvestigated. **Methods:** Conditioned medium from activated HMC-1 cells induces microglial activation similar to co-culture of microglia with HMC-1 cells. Primary cultured microglia were examined by flow cytometry analysis and confocal microscopy. TNF- α and IL-6 were measured with commercial ELISA kits. Cell signalling was analysed by Western blotting. **Results:** In the present study, we found that the conditioned medium from activated HMC-1 cells stimulated microglial activation and the subsequent production of the pro-inflammatory factors TNF- α and IL-6. Co-culture of microglia and HMC-1 cells with corticotropin-releasing hormone (CRH) for 24, 48 and 72 hours increased TNF- α and IL-6 production. Antagonists of histamine receptor 1 (H₁R), H₄R, proteinase-activated receptor 2 (PAR2) or Toll-like receptor 4 (TLR4) reduced HMC-1-induced pro-inflammatory factor production and MAPK and PI3K/AKT pathway activation. **Conclusions:** These results imply that activated mast cells trigger microglial activation. Interactions between mast cells and microglia could constitute a new and unique therapeutic target for CNS inflammation-related diseases.

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Introduction

Inflammation is fundamentally a protective cellular response aimed at the removal of injurious stimuli and the initiation of the repair process but can be a driver of disease when it fails to subside [1]. Several studies have demonstrated that neuroinflammation plays an

important role in the development of neurodegenerative diseases [2-4]. Recent progress suggested that the interaction between glia, immune cells, and neurons contributed to the exacerbation of acute symptoms of chronic neurodegenerative disease and accelerated disease progression. However, the microglia-immune cell connection has not been fully explored.

Microglia are the resident immune cells in the central nervous system (CNS) and secrete proinflammatory molecules that contribute to the pathogenesis of a broad range of CNS disorders. These disorders include not only infectious CNS diseases but also acute CNS injuries such as traumatic brain injury [5], spinal cord injury [6], stroke, and brain ischaemia [7]. Increasing evidence has demonstrated that the microglia-mediated neuroinflammatory process is critically involved in the initiation and development of neurodegenerative disorders such as Parkinson's disease (PD) [8], Alzheimer's disease (AD) [9] and multiple sclerosis [10]. When subjected to abnormal stimulation, such as neurotoxins, neuronal debris, or injury, microglia gradually become activated and produce a host of inflammatory factors. Thus, inhibition of microglial activation and the subsequent inflammatory process may identify novel therapeutic strategies to eliminate the deleterious effects of microglia.

Although microglial activation is widely recognized to participate in neuropathology, we must not ignore the finding that microglia also respond to proinflammatory signals released from other cells of immune origin, such as mast cells (MCs). MCs, which play a notorious role in allergic inflammation, are also found in the CNS where they are concentrated in the brain parenchyma along the blood vessels and leptomeninges [11, 12]. MCs produce a vast array of mediators, including proteases and vasoactive amines such as GnRH [13] and histamine [14]. MCs were reported to induce microglial activation and inflammatory mediator release in our previous studies [15, 16], suggesting the pivotal role of MCs in the induction of CNS inflammation. These neuromodulators released by MCs result in a variety of inflammatory diseases affected by stress [17]. Furthermore, meningeal MCs can recruit early T cells and neutrophils to the CNS, which in turn disrupt the blood-brain barrier [18]. The potential interactions between MCs and microglia, which may be involved in the pathobiology of neuroinflammation, have been reported to be the therapeutic target of neurodegeneration [19]. Yuan reported that mast cell activation induced microglia to release neurotrophin [20]. We previously reported that tryptase and histamine, which are mediators released from MCs, induced microglial activation and inflammatory mediator release [15, 16]. However, the effect of MCs on microglial activation is unclear. In this study, we investigated whether activated MCs directly evoked microglial activation *in vitro*.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were purchased from Gibco-BRL (Grand Island, NY, USA). The specific mouse anti-rat ED8 (anti-CD11b/CD18) monoclonal antibody (a marker for complement receptor 3 of activated microglia) was purchased from AbD Serotec (Raleigh, NC, USA). Fluoroshield mounting medium with 4, 6-diamidino-2-phenylindole (DAPI) was purchased from Abcam (HK, China). The Rat IL-6 Immunoassay Kit and Rat TNF- α Immunoassay Kit were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). The specific rabbit monoclonal antibodies against p38, phospho-p38, JNK, phospho-JNK, ERK, phospho-ERK, AKT, and phospho-AKT and the goat anti-rabbit secondary antibody were obtained from Cell Signalling (Beverly, MA, USA). The PE-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit IgG antibodies were purchased from BD (BD Biosciences, USA). The Toll-like receptor 4 (TLR4) neutralization antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA, catalogue number sc-10741). The histamine receptor 1 (H₁R) antagonist cetirizine dihydrochloride (cetirizine), H₂R antagonist ranitidine hydrochloride (ranitidine), H₃R antagonist carbinine dinitrofluoroacetate (carbinine), H₄R antagonist A943931 dihydrochloride (A943931), and NK-1 antagonist L-733060 were purchased from Tocris Bioscience (Bristol, UK). The proteinase-activated receptor 2 (PAR2) inhibitor FSLTRY-NH2 was synthesized by CL Bio-Scientific Inc. (Xi An, China).

Microglia-enriched cultures

Rat primary microglial cells were prepared according to the previously described protocol with slight modifications [21]. Briefly, whole brain tissues from postnatal (P1–P2) Sprague–Dawley rats were triturated. Then, the cells were plated onto poly-D-lysine precoated cell culture flasks in DMEM containing 10% foetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂/95% air. After obtaining a confluent monolayer of glial cells (10–14 days), the microglia were separated from the astrocytes by shaking for 5 h at 100 rpm and replated in 24-well culture plates at a density of 10⁵ cells/cm². The enriched microglia were >98% pure as determined by OX-42-IR.

HMC-1 Cell Culture

The human MC line HMC-1 was kindly provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN, USA). HMC-1 cells were cultured in DMEM culture medium supplemented with 10% foetal bovine serum, glutamine (2 µM) and penicillin-streptomycin (1:1000).

Co-culture of microglia and HMC-1 cells

Primary brain microglia (1 × 10⁶ cells) was grown in 25 cm² flasks until confluent. Then, HMC-1 cells (1 × 10⁶ cells) were added to the microglia flask because the MCs were floating cells. The cells were co-cultured for 12, 24, 48 and 72 hours.

Microglial challenge

Microglial cells were treated with conditioned medium from HMC-1 cells with or without corticotropin-releasing hormone (CRH) treatment for 12, 24, 48 and 72 hours and then incubated for an additional 24 hours.

Flow cytometry analysis

To determine the effect of HMC-1 cells on microglial activation, microglia were pelleted by centrifugation at 450 × g for 10 min and then fixed in 4% paraformaldehyde for 30 min. After washing, the cells were incubated with a PE-conjugated mouse anti-rat ED8 monoclonal antibody or isotype control (1:200) at 37°C for 1 h. Then, the cells were resuspended in PBS and analysed with a FACS Calibur flow cytometer with the CellQuest software (BD Biosciences, USA).

Immunofluorescence

To determine the effect of HMC-1 cells on microglial activation, microglia was fixed with 4% paraformaldehyde for 30 min. Nonspecific binding was blocked by incubating the cells in a 5% BSA and 0.1% Triton X-100 solution for 1 h at room temperature. The microglia were incubated with the monoclonal antibody ED8 (1:300) that recognizes complement receptor 3 (CD11b/CD18) overnight at 4°C. After three washes with PBS, the microglia were incubated with a PE-conjugated secondary antibody (1:200) and the nuclei were stained with DAPI. After three washes in PBS, the cells were smeared onto glass slides and the coverslips were sealed with nail polish. Fluorescent images were acquired using a confocal microscope (Zeiss).

TNF-α and IL-6 assays

The concentrations of TNF-α and IL-6 in the culture media were measured with commercial ELISA kits from R&D Systems.

Western Blotting

Microglia were collected and homogenized in 200 µl of lysing buffer. After incubation for 20 min on ice, the cell lysate was centrifuged and the protein concentration in the extracts was determined by the Bradford assay. Proteins (50 µg) in the cell extracts were denatured with sodium dodecyl sulfate (SDS) sample buffer and separated by 10% SDS–polyacrylamide gel electrophoresis. The proteins were transferred to PVDF membranes (Millipore) using a Bio-Rad miniprotein-III wet transfer unit. The membranes were incubated with 5% BSA dissolved in Tris-buffered saline with Tween 20 (TBST) (pH 7.5, 10 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h, followed by incubation of the membranes with

different antibodies overnight at 4°C. The following primary antibodies were used: rabbit monoclonal anti-c-Jun N-terminal kinase (JNK), -phospho-JNK, -p38, -phospho-p38, -ERK, phospho-ERK, -AKT, and -phospho-AKT (1:1000). After adding the goat anti-rabbit secondary antibody (1:1000) for 1 h, the protein bands on the membranes were detected with an enhanced chemiluminescence kit.

Statistical Analysis

All values are the means $\pm$ SEM. The significance of the differences between the controls and samples treated with various drugs was determined by one-way ANOVA followed by the post-hoc least significant difference test. Differences were considered significant at $P < 0.05$.

Results

CRH induced HMC-1 activation and mediator release

The human leukaemic MC (HMC-1) cell line expresses functional CRH receptors and can be activated by CRH [22, 23]. Therefore, we investigated whether CRH induced pro-inflammatory factor production from HMC-1 cells. As shown in Fig. 1A, stimulation of HMC-1 cells with CRH caused concentration-dependent ($1-10^4$ nmol) and time-dependent (> 24 h) HMC-1 activation indicated by the increase in the Fc-positive cell number. However, CRH treatment ($1-10^4$ nmol) for 12 h had no effect on HMC-1 activation. We also found that incubation with CRH (100 nmol) for more than 48 hours resulted in the production of TNF- α and IL-6 by the HMC-1 cells (Fig. 1B). These results indicate that long-term CRH treatment (> 48 h) can induce HMC-1 activation and pro-inflammatory factor production.

Fig. 1. CRH induced HMC-1 degranulation. (A) HMC-1 cells were treated with CRH ($1-10^4$ nmol) for 24, 48 and 72 h, resulting in increased numbers of Fc-positive cells. (B) CRH (100 nmol) induced the release of TNF- α and IL-6 from HMC-1 cells following more than 48 h of incubation. * $P < 0.05$, ** $P < 0.01$ vs. the control HMC-1 group. The data are presented as the mean $\pm$ SEM of four independent experiments.

Fig. 2. Activated HMC-1 cells increased microglial activation and TNF- α and IL-6 production from microglia. Primary microglia were treated with CM from HMC-1 cells with or without CRH stimulation for 24 hours for different incubation times (12, 24, 48 and 72 h). (A) For the flow cytometry analysis, the cells were incubated with a PE-conjugated ED8 antibody at 37°C for 1 h. (B) Cells were stained with an ED8 antibody, and upregulated ED8-immunopositive expression (red) on activated microglia was observed using confocal microscopy. The blue staining represented DAPI. Scale bar: 50 μ m.

Activated HMC-1 cells induced microglial activation

Activated microglia were detected with the monoclonal antibody ED8, which recognizes complement receptor 3 (CR3). Microglia were incubated for 12 hours with CM from HMC-1 cells with or without CRH-stimulation (12, 24, 48 and 72 hours). The flow cytometry analysis showed that approximately 33.9% and 46.6% of the microglia were activated by incubation in the CM from HMC-1 cells stimulated with CRH for 48 and 72 hours, respectively. However, the CM from HMC-1 cells stimulated with CRH for 12 and 24 hours had no effect on microglial activation (Fig. 2A). A 24 h incubation with the CM from HMC-1 cells stimulated with CRH for 48 and 72 hours resulted in a remarkable upregulation of ED8 expression (in red, Fig. 2B).

Fig. 3. (A) Incubation of microglia with CRH (100 nmol) for more than 48 h inhibited TNF- α and IL-6 production. (B) Incubation of microglia with CM from HMC-1 cells had no effect on TNF- α and IL-6 production, but the CM from CRH-stimulated HMC-1 cells (48 and 72 hours) induced the release of TNF- α and IL-6 from microglia following 24 h of incubation. * $P < 0.05$, ** $P < 0.01$ vs. the control microglia group. The data are presented as the mean $\pm$ SEM of four independent experiments.

However, the CM from HMC-1 cells without CRH stimulation had no effect on ED8 expression (Fig. 2B). These results suggested that mediators released from activated HMC-1 cells could activate microglia.

Activated HMC-1 cells induced TNF- α and IL-6 production by microglia

Because microglia-mediated neuroinflammation primarily occurs through the excessive production of pro-inflammatory and cytotoxic factors from activated microglia and their downstream signalling cascades, we measured the levels of pro-inflammatory factors. As shown in Fig. 3A, incubation with CRH (100 nmol) for 12, 24, 48 and 72 hours did not increase the production of TNF- α and IL-6; indeed, incubation for 48 and 72 h decreased the production of TNF- α and IL-6. Incubation of microglia with the CM from HMC-1 cells without CRH had no effect on TNF- α and IL-6 production, whereas the CM from CRH-stimulated HMC-1 cells (48 and 72 hours) induced the release of TNF- α (172 and 326 pg/ml) and IL-6 (669 and 1599 pg/ml) from the microglia following 24 h of incubation (Fig. 3B).

To confirm that activated HMC-1 cells induced the release of pro-inflammatory factors from microglia, we co-cultured microglia with HMC-1 cells. As shown in Fig. 4, microglia and HMC-1 cells were co-cultured with or without CRH (100 nmol) for 12, 24, 48 and 72 hours. The co-culture of microglia and HMC-1 cells with CRH for 24, 48 and 72 hours resulted in increased TNF- α and IL-6 production compared with the culture of microglia alone.

The effect of receptor antagonists on HMC-1-induced microglial activation

MCs contain an array of chemical mediators that can be released to the micromilieu upon activation. To elucidate which mediators were involved in the promotion of microglial activation, we selectively blocked the histamine receptors PAR2, NK-1 and TLR4 in MCs [15,

Fig. 4. Co-culture of microglia with HMC-1 cells. The TNF- α and IL-6 levels in the CRH co-culture group were higher than the other groups after 24 h of incubation. * $P < 0.05$, ** $P < 0.01$ vs. the control microglia group. The data are presented as the mean $\pm$ SEM of four independent experiments.

Fig. 5. Antagonists of H_1R , H_4R , PAR2 or TLR4 partially inhibited HMC-1-induced TNF- α and IL-6 production in microglia. CM from CRH-stimulated HMC-1 cells (48 h) was incubated with microglia at 37°C for 24 h. ** $P < 0.01$, * $P < 0.05$ vs. the control microglia group. # $P < 0.05$ vs. CM from CRH-stimulated HMC-1 treatment group. The data are presented as the mean $\pm$ SEM of four independent experiments.

16]. As shown in Fig. 5, the H_1R antagonist cetirizine (10 μ M), H_4R antagonist A943931 (10 μ M), PAR2 inhibitor FSLRY-NH2 (400 μ M) and TLR4 neutralizing antibody (10 μ g/ml) partially abolished the ability of CM from CRH-stimulated HMC-1 cells to induce TNF- α and IL-6 release from microglia. Conversely, the H_2R antagonist ranitidine (10 μ M), H_3R antagonist carbinine ditrifluoroacetate (10 μ M) and NK-1 antagonist L-733060 (100 μ M) had little effect on the ability of activated HMC-1 cells to induce TNF- α and IL-6 production from microglia. These results indicated that activated HMC-1 cells induced TNF- α and IL-6 production from microglia partially through the activation of H_1R , H_4R , PAR2 and TLR4.

The MAPK and AKT pathways mediated MC-induced microglial activation

We found that the MAPK and AKT signalling pathways were involved in MC-induced microglial activation *in vivo* [24]. To confirm this finding, we investigated these signalling pathways *in vitro*. Treatment with CM from CRH-stimulated HMC-1 cells (48 h) led to rapid and transient phosphorylation of ERK, p38, JNK and AKT that was indicative of ERK, p38, JNK and AKT activation, with the peak levels of phospho-ERK occurring at 60 min, phospho-p38 occurring at 15 min, phospho-JNK occurring at 120 min and phospho-AKT occurring at 120

Fig. 6. Effects of the H_1R , H_4R , PAR2 and TLR4 antagonists on HCM-1-induced MAPK and PI3K/AKT activation. (A) CM from CRH-stimulated HMC-1 cells (48 h) activated ERK, p38, JNK and AKT as assessed by the increased phosphorylation of the tyrosine residues of these kinases. (B) The H_1R and H_4R antagonists suppressed the ability of CM from CRH-stimulated HMC-1 cells (48 h) to induce p38, JNK and AKT activation in microglia. The PAR2 antagonist suppressed the ability of the CM from CRH-stimulated HMC-1 cells (48 h) to induce ERK, JNK and AKT activation in microglia. The TLR4 antagonist suppressed the ability of the CM from CRH-stimulated HMC-1 cells (48 h) to induce ERK, p38, JNK and AKT activation in microglia. Microglia were pretreated with the antagonists for 30 min and then exposed to CM from CRH-stimulated HMC-1 cells (48 h) for 60 min (p-ERK), 15 min (p-p38), 120 min (p-JNK) or 120 min (p-AKT). * $P < 0.05$, ** $P < 0.01$ vs. the control microglia group. # $P < 0.05$ vs. CM from the CRH-stimulated HMC-1 treatment group. Data are presented as the mean $\pm$ SEM of three independent experiments.

min (Fig. 6A). These data indicate that the MAPK and AKT signalling pathways are involved in HCM-1-induced microglial activation. As shown in Fig. 6B, the TLR4 neutralizing antibody (10 μ g/ml) suppressed the ability of HCM-1 cells to induce the activation of all three mitogen-activated protein kinases (MPAK) and AKT phosphorylation in microglia. However, the H_1R antagonist cetirizine (10 μ M) and H_4R antagonist A943931 (10 μ M) suppressed JNK, p38 and AKT phosphorylation and the PAR2 inhibitor FSLRY-NH2 (400 μ M) suppressed ERK, p38 and AKT phosphorylation in microglia. These results indicate that HCM-1 activates the MAPK and AKT signalling pathways via H_1R , H_4R , PAR2 and TLR4 in microglia.

Discussion

Pathological mechanisms that regulate neuroinflammation, which plays an important role in provoking the occurrence and development of neurodegenerative diseases, may prove to be a useful therapeutic target for central nervous system disorders. This finding also raises the question of whether we are missing important therapeutic avenues by studying microglia and MCs in isolation [19]. Our previous study showed that the neuromediators histamine and SP triggered microglial activation, indicating the possibility of communication between MCs and microglia [25]. However, direct evidence concerning whether MCs could affect microglial activation was lacking. In this study, we found that activated MCs induced TNF- α and IL-6 production from microglia partially via H₁R, H₄R, PAR2, and TLR4-MAPK. These results may provide novel therapeutic targets for the treatment of neuroinflammation.

Emerging evidence now points to the irremissible responsibility of neuroinflammation in pathophysiology onset and progression, with microglia playing key roles in neurodegenerative diseases such as PD [26, 27], AD [28-31], amyotrophic lateral sclerosis [32], and possibly even depression, schizophrenia, and other psychiatric disorders [33, 34]. Our previous *in vitro* study suggested that high glucose augmented LPS-induced microglial activation and inflammatory cytokine levels, which offered new insight into the pathophysiological relationship between diabetes mellitus and POCD [35]. Our *in vivo* study showed that S100A8 resulted in microglial activation following the occurrence and development of surgery-induced neuroinflammation and cognitive dysfunction [36]. Activated microglia can release BDNF, which causes prolonged microglial activation [37]. Therefore, the inhibition of microglial activation and subsequent neuroinflammation may offer prospective clinical therapeutic benefits for neuroinflammation-related neurodegenerative disorders. However, the factors responsible for the overactivation of microglia are largely undefined.

A previous study suggested that activated MCs but not microglia were the “first responders” in brain injury [38]. Although other resident cells in the CNS produce TNF- α (most notably microglia [39, 40] and endothelial cells [41]), the presence and release of TNF- α from MCs precedes its detection in other cells. Recent *in vivo* studies indicated that MCs actively participated in the pathogenesis of inflammation through the release of proinflammatory mediators. If early MC activation is necessary for the initiation of the inflammatory cascade and ultimately tissue damage, the inhibition of this response should be neuroprotective. In the present study, we found that only CRH-stimulated HMC-1 cells activated microglia and induced TNF- α and IL-6 release. HMC-1 is an immature MC line that can be activated by CRH [22, 23]. We found that incubation with CRH (100 nmol) for more than 48 hours resulted in the activation of HMC-1 cells and the production of TNF- α and IL-6. Conversely, HMC-1 cells cultured in the absence of CRH had no effect on microglial activation. These results confirm that only activated MCs can induce microglial activation and pro-inflammatory factor release.

The factors responsible for the overactivation of microglia are largely undefined. Several molecular mechanisms for potential communications between MCs and microglia have been determined *in vitro* [19]. For instance, ATP can stimulate the activation of microglia through purinergic P2 receptors and the subsequent release of IL-33. IL-33 binds to MC receptors and triggers the production of monocyte chemo-attractant protein 1, IL-6 and IL-13, which in turn may regulate microglial activity. Similarly, tryptase released from MCs activates the microglial PAR-2 receptors, thereby initiating the production of inflammatory mediators such as TNF- α , IL-6, and ROS that consequently upregulate the expression of PAR2 receptors on MCs [15, 42]. Our previous studies showed the presence of tryptase and histamine receptors on the surface of microglia. Tryptase can induce microglial activation and proinflammatory mediator release via the protease-activated receptor 2 (PAR2)-MAPK-NF-kappa B signalling pathway, whereas histamine induces TNF- α and IL-6 release from activated microglia via the H₁R, H₄R-MAPK and PI3K/AKT-NF-kappa B signalling pathways [15, 16]. Here, we demonstrate that mediators released from activated MCs can induce microglial activation through H₁R, H₄R, PAR2 and TLR4 in the microglia. The antagonists of H₁R, H₄R,

PAR2 and TLR4 inhibited activated HMC-1-induced microglial activation in our *in vitro* study. The MAPK and AKT signalling pathways were involved. All of the above results imply that activated MCs trigger microglial activation and subsequent neuroinflammation. Therefore, the communication of MCs with microglia could constitute a new and unique therapeutic target for CNS immune inflammation-related diseases.

In conclusion, to the best of our knowledge this is the first study to demonstrate the direct effect of MCs on microglia and the involved signalling pathways. Activated MCs can trigger microglial activation via H₁R, H₄R, PAR2 and TLR4, which suggests that interactions between MCs and microglia may constitute a new and unique therapeutic target for CNS immune inflammation-related diseases.

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Disclosure Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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