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Methionine enkephalin regulates microglia polarization and function

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ABSTRACT

Methionine enkephalin (MENK), an opioid peptide, is known to function as a regulator in the immune system. As microglia are considered the most important immune cells in the central nervous system (CNS), we aimed to assess the function of MENK on microglia polarization and tumoricidal responses. Initially, we chose the most optimal condition of 10^{-12} M for 48 h; however, MENK had no function on the viability and apoptosis of microglia under this treatment. However, MENK treatment markedly increased levels of M1-associated genes, such as CD86, CD40, IL-12, and TNF-α, but had no effect on M2 markers, including CD163, IL-10, and TGF-β. Moreover, microglia in the MENK-treated group showed high phagocytosis capacity, which coincided with characteristics of M1 microglia. MENK stimulation also induced up-regulation of reactive oxygen species (ROS) expression, which contributed to maintaining homeostasis. We also detected NO production by measuring the end product nitrite, and found that MENK treatment increased expression of nitrite and inducible NO synthase (iNOS), but did not influence arginase-1 (Arg1) expression. Furthermore, treatment of microglia with MENK led to a significant increase in cytotoxicity against glioblastoma cells, indicating that MENK possessed anti-tumor ability. Overall, MENK treatment could induce microglia to an M1 phenotype, modulating Th1 responses in the immune system. Additionally, microglia treated with MENK had tumoricidal activity, which provides new insight into anti-tumor immunity.

1. Introduction

Binding to various opioid receptors, methionine enkephalin (MENK) regulates various functions in organisms, including neuron protection, tumor inhibition, wound healing, cell therapy, and angiogenesis [1–3]. Our research team has found that, as an endogenous opioid peptide, MENK functions as an immunomodulator, stimulating various immune cells such as T lymphocyte cells, dendritic cells (DCs), macrophages, and natural killer cells [4–7]. Additionally, MENK also inhibits tumor growth and takes part in anti-tumor immunity [2,8].

As the most important immune cells in the central nervous system (CNS), microglia monitor the microenvironment and defense pathogens, and combat infectious agents when necessary, playing a vital role in maintaining brain homeostasis [9–11]. Under normal conditions, microglia exhibit the resting phenotype, which are charactered with the low expressions of CD45, co-stimulatory molecules, and MHC II [12–14]. After encountering pathogens, resident microglia become sensitized, and activated microglia become phenotypically undistinguishable from macrophages [13]. As the counterpart to macrophages, microglia can be activated by various stimulations and polarized into a classically activated (M1) or alternatively activated (M2) phenotype. M1 microglia, activated by interferon-γ (IFN-γ), lipopolysaccharide (LPS), and toll-like receptor (TLR) agonists, express activation markers (such as CD40 and CD86), proinflammatory molecules (such as TNF-α and IL-12), cytotoxic factors (such as nitric oxide (NO), inducible NO synthase (iNOS) and reactive oxygen–nitrogen species (ROS)) [15–17]. On the other hand, M2 microglia can be polarized by IL-4 or IL-13, and secrete surface molecules (such as CD163), anti-inflammatory molecules (such as IL-10 and TGF-β), and express high levels of arginase-1 (Arg1) [18,19]. Due to secreted molecules, M1 microglia are involved in tissue defense, inducing cytotoxicity to pathogens and tumor cells, promoting Th1 polarization, and protecting the body from destruction by pathogens. In contrast, M2 microglia can maintain homeostasis by healing wounds, remodeling tissue, scavenging debris, enhancing neuronal survival, and reducing neuronal death. Our research team has found an important role for MENK in immune responses, whereas little is known about its role in microglia. Therefore, we aim to examine the role of

Abbreviations: Arg1, arginase-1; CNS, central nervous system; CAMK, Ca^{2+}/calcineurin-dependent protein kinase; DCs, dendritic cells; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; FCM, flow cytometry; IFN-γ, interferon-γ; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MENK, methionine enkephalin; NO, nitric oxide; OD, optical density; ROS, reactive oxygen–nitrogen species; TLR, toll-like receptor.

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MENK on microglia polarization and homeostasis to gain further insight into the function of this peptide in the immune system.

2. Materials and methods

2.1. Reagents

MENK (>97% purity) was obtained from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). Recombinant IFN-γ and IL-4 were from PeproTech Inc. (Rock Hill, NJ, USA). LPS and FITC-labelled dextran were from Sigma Aldrich, Inc. (St. Louis, MO). The PrimeScript™ RT reagent Kit, TRIzol Reagent, and SYBR® Premix Ex Taq™ II kit were obtained from Takara. (Tokyo, Japan). The Annexin V-FITC Detection Kit was obtained from Biotool (Houston, Texas, USA). The enzyme-linked immunosorbent assay (ELISA) kits for human IL-12, TNF-α, IL-10, and TGF-β were obtained from eBioscience Inc. (San Diego, CA, USA). The mAbs for flow cytometry (FCM), including anti-CD86, anti-CD163, anti-CD40 and isotype-matched Ab, were obtained from Biolegend Inc. (San Diego, CA, USA). NO, Crystal Violet Staining Solution, and Reactive Oxygen Species assay kits were obtained from Beyotime Biotechnology Co. (Jiangsu, China). The MTS solution was obtained from Promega (Madison, WI, USA).

2.2. Primary microglia culture

Human microglia were obtained from ScienCell Research Laboratories (San Diego, CA, USA), and U87 glioblastoma were from the Shanghai Institute of Biochemistry (Shanghai, China). The microglia are isolated from primary human fetal brain cell culture and are characterized by immunofluorescence with antibodies to CD11b/c. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum under normal growth conditions (typically 37 °C and 5% CO_2). In our experiments, microglia were digested into cell suspensions and seeded onto 96- or 6-well plates. As IFN-γ dramatically increases the responsiveness to LPS on microglia [20], we used LPS (100 ng/ml) plus IFN-γ (20 ng/ml) to generate M1 microglia. To generate M2 microglia, IL-4 (20 ng/ml) was added to the culture medium.

2.3. ELISA to find the optimum concentration of MENK

To determine optimum treatment conditions, we detected IL-12 expression by microglial cells by altering the concentration of MENK from 10^{-15} to 10^{-8} M for 24 and 48 h. Microglia were incubated on a 96-well plate at a density of 2×10^5/ml (220 μl/well), and cells were cultured in DMEM alone as a control. After incubation, the supernatant was collected and IL-12 expression was detected by ELISA following manufacturer's instructions. Results were measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.4. Microglia viability by MTS assay

Microglia were incubated on a 96-well plate at the final density of 2×10^5/ml (200 μl/well) with optimal drug concentrations for 48 h. The MTS assay was used for measuring microglia viability, and absorbance at 490 nm was read by a microplate reader.

2.5. Microglia apoptosis by FCM

Microglia were incubated on a 6-well plate at a density of 3×10^5/ml (3 ml/well) with optimal drug concentrations for 48 h. After treatment, microglia were rinsed and detached in PBS. FITC Annexin V and PI were added into cell suspension, which was incubated for 15 min at 25 °C in the dark. After that, stained microglia were assessed by a Gallios flow cytometer (Beckman Coulter, CA, USA) and results were analyzed by FlowJo software.

2.6. Cell surface proteins by FCM

Microglia were incubated on a 6-well plate at a density of 3×10^5/ml (3 ml/well) with optimal drug concentrations for 48 h. After that, cultured cells were collected and detached in cold binding buffer, and cells were surfaced-stained with CD86, CD40, CD163 mAbs, or isotype-matched Abs for 30 min at 4 °C. Antibody concentrations were performed strictly according to the manufacturer's protocol. Then, stained cells were resuspended in binding buffer, and the expression level was detected by a Gallios flow cytometer and FlowJo software, counting the ratios of positive cells.

2.7. Cytokine measurement by ELISA

Microglia were incubated on a 6-well plate at a density of 3×10^5/ml (3 ml/well) with optimal drug concentrations for 48 h. After that, the concentrations of IL-12, TNF-α, IL-10, and TGF-β in supernatants were assessed by ELISA following manufacturer's instructions. Data were detected by a microplate reader.

2.8. Real-time PCR

Microglia were incubated on a 6-well plate at a density of 3×10^5/ml (3 ml/well) with optimal drug concentrations for 48 h. After that, total RNA was isolated from microglia, and complementary DNA was synthesized by RT reagent Kit. PCR was performed on an ABI PRISM cycler (Applied Biosystems, Foster City, CA), and the primers were shown in Table 1. Relative mRNA expression levels were analyzed by the comparative $2^{-\Delta\Delta Ct}$ formula and data were normalized by a housekeeping gene (*β-Actin*).

2.9. Phagocytic capacity by FCM

The uptake of FITC-dextran was used to measure the phagocytic ability. Microglia were incubated on a 6-well plate at a density of 3×10^5/ml (3 ml/well) with optimal drug concentrations for 48 h. After that, cultured cells were incubated with a final concentration of 1 mg/ml of FITC-labelled dextran for 30 min at 4 °C. After washing, samples were detected by a Gallios flow cytometer, and data were analyzed by FlowJo software.

2.10. Measurement of intracellular ROS in microglial cells

Microglia were incubated on a 6-well plate at a density of 3×10^5/ml (3 ml/well) with optimal drug concentrations for 48 h. After that, cultured microglia were treated with a final concentration of 10 nmol/ml of 2,7-dichlorodihydrofluorescein diacetate for 20 min under normal growth conditions, and washed using serum free medium. After that, microglia were detached in PBS, and samples were measured by a

Table 1
The primers of genes.

Gene	Sequence
β-Actin	5′-TGGCACCCAGCACAATGAA-3′
	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′
IL-12	5′-GTCACAAAGGAGGCGAGGTT-3′
	5′-ACTGATTGTCGTCAGCCACC-3′
TNF-α	5′-TCCTCACCCACACCATCA-3′
	5′-GGAAGACCCCTCCCAGATAG-3′
IL-10	5′-CATCAAGGCGCATGTGAACT-3′
	5′-GATGTCAAACTCACTCATGGCTTT-3′
TGF-β	5′-GACTGCGGATCTCTGTGTCAT-3′
	5′-AGTAGTGTTCCCCACTGGTCC-3′
iNOS	5′-CATCCTCTTTGCGACAGAGAC-3′
	5′-GCAGCTCAGCCTGTACTTATC-3′
Arg-1	5′-CTACAAAACAGGGCTACTCTCAGGAT-3′
	5′-GTTCGAGTTACTTCTTCTGGTGTCTTC-3′